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On the occasion of Professor Knud O Møller's 70th birthday Acta Pharmacologica et Toxicologica and its editors have presented him with this wood-cut as a token of their admiration and good wishes.

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From the Department of Pharmacology of H. Lundbeck & Co. A/S,
Ottiliavej 7 Copenhagen-Valby

Pharmacology of a New Antitussive Agent Meprotixol (N 7020)

By

W Haegs, I Møller Nielsen, S. Norn and M. Nymark

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In a previous paper (MOLLER NIELSEN *et al* 1962) a comparison of the central depressive activity of 66 thiaxanthene derivatives with those of 8 phenothiazines was reported.

Among the thiaxanthenes 2-methoxy 9(dimethyl-aminopropyl)-thiaxanthenol (N 7020 meprotixol pINN) (fig. 1) was found to have only a slight central depressive effect.

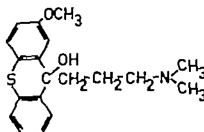


Fig. 1 Formula of meprotixol (N 7020).

Further studies of this compound revealed a potent antitussive activity This paper describes its pharmacological properties

Methods

Meprotixol was available as the base, a white substance (mol.wt. 329.4), soluble in water on the addition of equivalent amounts of acid. All results given in this paper refer to the base. As control substances the drugs used in the form of salts were codeine phosphate, chlorpromazine-HCl, chlorprothixene-HCl and clopenthixol-2HCl. In presenting the results, these compounds will be referred to without mention of the salt.

M tility in mice

The spo taneous motor ctivity of mice was determined i jiggle cages by the method previously described (MØLLER NIELSEN & NEUHOLD 1959) The DR50 is the dose that reduces the activity (15 minute test-period) of treated mice to half that of control mice (1 animals per group).

Potentiatio of barbiter te anaesthesia i mice

a) Two groups f mice (= 10) were pretreated, one with 2.5 the other with 25 mg/kg meprotixol i.p. An intravenous injection of 50 mg/kg enhexymal-Na (hexobarbital sodium) was given 30 min tes later Sleeping time, defined as the time during which the animals remai on their backs, was recorded. The potentiating factor is defined as

$$\frac{\text{sleeping time, test animals}}{\text{sleeping time control animals}}$$

b) Four groups of 10 mice each were pretreated intraperitoneally with increasing doses of meprotixol Half a hour later 50 mg/kg enhexymal-Na were injected intraven usly the sleepi g time was recorded nd compared with that of control mice. Observatio time was limited to 30 minutes thus > preceding the verage sleeping time for the group (I T ble 3) indicates that at least one mouse slept for longer than 30 minutes.

c) The duratio of th enhexymal potentiating effect was determined by pretreati g 5 groups of 10 mice each with meprotixol, 25 mg/kg i.p At various intervals after the meprotixol dosage, the gro ps were given i travenous i jections f enhexymal-Na, 50 mg/kg. The sleepi g times f treated mice were compared with that of control mice.

d) Three groups of mice (= 10) were given enhexymal i. and the sleeping time was recorded. At the time of awakening the mice wer given n intravenous injection of small doses f meprotixol that would t in themselves i duce sleep. If potentiation occurs, the animals will gai become anaesthetized. The second sleeping time is recorded.

) G ups of 10 mice were pretreated with meprotixol i p. 30 minutes before divided intravenous injection f thi mebumal-Na (thiopental sodium), 0 25 %, 0 05 ml every 10 seconds until anaesthesia ensued. Sleeping times d volumes of anaesthetic dose f thiomebumal solution were recorded.

Potentia ion of alcohol naesthesia in mice

Gro ps f 10 mice were pretreated with meprotix l i.p. 30 minutes before n intravenous injecti n f 20 solutio of ethanol i jected in divided doses f 0 05 ml every 10 seconds until anaesthesia occurred. The volumes of anaesthetic doses of alcohol and sleepi g times were registered.

Effect on body temperature on rats

The body temperature of individually caged restrained rats was recorded by means of a thermoco ple. (Room temperature 22–24) The temperature was measured 15 mi utes before dosing and ¼ ½ 1 1½ ., 3 4 and 5 hours after i.p. injection of the test compound.

Anticataleptic effect ertical rod test on mice

A vertical iron rod, diameter 15 mm, height 80 cm, was wound with string. The experimental mice were divided int groups of 10. N rmal mice placed on the rod will imme-

diately climb up or down it. Mice treated with suitable doses of a neuroleptic will remain where placed for more than 30 seconds (cataleptic reaction). When pretreated with a drug having anticataleptic action before the injection of the neuroleptic, the mouse will climb the rod like the untreated mouse (ZETLER 1963). The experimental procedure was to give the test compound s.c. 30 minutes later a cataleptic dose of chlorprothixene (2.5 mg/kg s.c.) was injected. 20 minutes after the injection of chlorprothixene the animals were tested on the rod every 5 minutes for one hour (a total of 13 trials).

Evaluation	Mouse climbs within 30 seconds	—
	Mouse does not climb within 30 seconds	+
	Mouse falls or slides from rod is marked (+) but counts as	+

Average of + (cataleptic reaction) per group was calculated. (Maximal possible score 13).

Antireserpine effect in mice

Injection of reserpine, 2 mg/kg i.p., into mice causes increasing sedation and ptosis. The ptosis was evaluated 30, 60, 90 and 120 minutes after injection, on an arbitrary scale from 0 to 4. The score 0 is given for fully opened eyes; 4 means maximal closure of eyelids. Compounds with antagonistic action against the central depressive effect of reserpine will delay or prevent the development of reserpine-induced ptosis. The compound is given i.p. 30 minutes before administration of reserpine.

Influence on $\overline{LD100}$ of convulsive and other toxic agents

Mice of body weights from 18 to 25 g were employed. For the individual experiments the mice were selected so that the weight difference between animals did not exceed 2 g. Groups of 10 mice were pretreated with test compound before the injection of the convulsive/toxic agent, which was injected in solution of suitable concentration, at a rate of 0.05 ml every 10 seconds until convulsions or death occurred, or both. The average volume of toxic solution ($\overline{LD100}$) was recorded. The $\overline{LD100}$ values for test animals were compared with that of untreated control animals (HINT & RICHTER 1958). When the former is larger than the latter the test compound has protective action against the toxic agent in question. Meprotixol has been tested against the lethal or convulsive action of the toxic agents harmine, nicotine, picrotoxin, mescaline, acetylcholine, strychnine and pentetrazol.

Protection against electroconvulsions in mice

Electroconvulsions are induced in mice with eye electrodes (SWINYARD 1949). A high voltage current, 9.5 mA, 0.2 sec induces maximal electroconvulsive seizure (MES) with full tonic extension of all limbs in 100 % of mice. Groups of 10 mice were pretreated with the test compound 30 minutes before challenging by electrostimulation. The PDMES50 the dose protecting 50 % of mice against the maximal electroconvulsive seizure (end-point, tonic extension of limbs), was recorded.

Effect on righting reflex in rats

Attempts are made to place the rats, pretreated with the test compound, on their back. Rats with unimpaired righting reflex will immediately get on to their feet. Pretreated with anaesthetics or neuroleptics in suitable doses, rats may remain on their backs for more than 30 seconds. The ED50 was determined.

Holding reflex, rotating drum-test on mice

Mice are placed in an inclined wire-mesh cylinder (diameter 20 cm length 70 cm, mesh 0.5 × 0.5 cm). The cylinder was raised at 60° angle and rotated at 1½ revolutions per minute. Ten mice were placed in the cylinder Every 15 mi utes the number of mice that have fallen down from the cylinder is recorded. The ED50 was determined in the usual manner

Balance test otating rod on rats

Rats were placed on a horizontally rotating wooden rod, diameter 22 mm, 3 rotations per mi ute. Groups of 10 animals were employed, and the percentage of rats unable to stay on the rod for 15 seconds was recorded. The ED50 was determined.

Antitussi e action on th anaesthetized cat

The antitussive effect was determined in cats by the method of DOMENJOZ (1952). T cats, anaesthetized with an intraperitoneal injection of 70 mg/kg hypnophen Ph. Dan. 48 (allypropymal + diallymal, equal parts), as required additional doses of hypnophen were given ntravenously the superior laryngeal nerve was dissected free and stimulated every 5 min tes (current 5–10 Hz, 0.2–1 msec. 1–5 volt, duration 5–10 seconds). Each stim lation would give rise to a reproduc ble cough response, which was recorded kymographically via a pharyngeal tube and a tambour

Influenc espiration

In cats naesthetized with hloralose urethane r hypnophen the respiratory volume was recorded by means f a spirometer (GILDEMEISTER 1922) the amplitude with tambour and the frequency with mercury contact mounted on the lever

Influence on potential waves derived from the phren nerve

Cats were anaesthetized with chloralose-urethane and tracheotomized. A bipolar platinum recording electrode was placed centrally on the cut phrenic nerve. The impulse waves, which were amplified (Amplifier S & W A 59) and recorded by means of an oscilloscope (DISA 51BOO) equipped with 35 mm camera, are considered to be an expressio of the activity of the respiratory centre (HOUGS 1964).

I fluence on blood pressure

Experiments were carried out on rats under urethane anaesthesia d on cats under chloralose-urethane naesthesia In both species the blood pressure was recorded by cannula in the carotic or femoral artery with a co ventional mercury manometer and writing on smoked drum.

Electrocardiographi alues

The ECG values were corded from naesthetized cats (chloralose-urethane or hypnophen) and rabbits under nitrous oxide/oxygen anaesthesia. Recordings were made with an oscilloscript (Philips), with 3 leads, bef re and 5 10 and 15 minutes fter intravenous injection of meprot xol

Autonomic effects on rabbits

In rabbits anaesthetized with urethane the effects of meprotixol on blood pressure, on respiration and on the motility of jejunum and uterus in situ were studied. The tonus and contractions of uterus and gut were recorded kymographically by means of isotonic myographs and frontal writing levers.

Influence on the function of the nictitating membrane in the cat

In cats anaesthetized with chloralose-urethane stimulating electrodes were placed on the cervical sympathetic trunk on the preganglionic as well as on the postganglionic fibres. The nictitating membrane was connected by means of a string with a light myograph (counterweight 5 g). The contractions of the membrane in response to submaximal preganglionic and postganglionic stimulation (10 Hz, 0.2–4 1 msec., 10 sec.) of the nerve as well as to intravenous injection of noradrenaline (10 µg/kg i.) were recorded. These procedures were repeated in the sequence mentioned at 5 minutes intervals, one cycle being completed each 15 minutes

Adrenaline, noradrenaline and serotonin antagonism

The antagonisms against adrenaline, noradrenaline and 5-hydroxytryptamine pressor responses in pithed rats were studied by the method previously described by MØLLER NIELSEN & NEUHOLD (1959).

Acetylcholine antagonism

The effect of meprotixol the acetylcholine-evoked contractions of the isolated guinea pig ileum was determined by the method of MAGNUS (1904).

Effect on pupil diameter in mice

The pupil diameter in mice was measured an arbitrary scale in binocular dissecting microscope under constant lighting conditions. The dose causing 100 % increase in pupil diameter was determined.

Broncholytic effect guinea pig

The broncholytic effect of meprotixol was studied in modified Konzett Rössler preparation (AUKRUP & HOUGS 1962). Bronchospasms were elicited by intravenous injections of acetylcholine or 5-hydroxytryptamine.

Antinociceptive activity

Four test methods were used for determining the action of meprotixol on nociceptive stimuli in mice.

) Haffner's test (tail pinching) (HAFFNER 1929). Mice were pinched at the root of the tail with Péan. End point squeaking or biting after the Péan. Each mouse was tested 15 30, 45 and 60 minutes after the injection. The ED50 is the dose eliminating this reaction in 50 % of the mice

b) Phenylquinone-test. I traperitoneal injection int mice of a 0.03 / solution of phenylquinone causes a series of writhing movements attributed to abdominal pai The animals were observed for a period of 12 mi utes after the injectio of phenylquinone. The ED50 is the dose of test compound that prevents the phenylquinone reaction within the experimental period (12 minutes) in 50 / of the mice.

c) Hot plate test. Mice were placed in a perspex cylinder on a copper plate heated to 58 Normally the animals react to this heat stimulus by lifting their paws, licking their paws o jumping out of the cylinder The animals were tested before treatment and their reaction times were recorded. After injection of the test compound the animals were retested at 15 30, 45 and 60 minutes. The ED50 was determined as the dose that bolished the reac tion in a test period of 30 seconds I half of the animals.

d) Electrical stimulation of the tail. Mice were placed in narrow wire-mesh cylinder and two needle electrodes, 8 mm part, were placed subcutaneously at the root of the tail. Electrical stimuli (square wave, 1 Hz, 20 msec., 4 sec.) were pplied through the electrodes with increasing volt ge until a squeak was heard. The voltage required for this reaction was defined as the threshold. The threshold of untreated animals varied from 2 to 8 volts. After injection of the test compound the threshold was redetermined 15 30, 45 and 60 minutes later The ED50 was the dose that in 50 of the animals doubled the threshold.

In all antinociceptive tests 10 mice per dose level were used.

Local anaesthetic effect on mic

The reflex to stimulation of the cornea with Frey hair was used for determining sur face naesthesia. Solutions of test compound at various concentrations were instilled into the conjunctival sac. The ED50 was defined as the concentration inhibiting the corneal reflex in 50 % of the animals.

Antiphlogistic effect o rats

Four test methods were used to assess the antiphlogistic ction of meprotixol.

a) Rat paw oedema was induced in the hind paws by subcutaneous injectio (plantar surface) of various phlogistic gents dextran 0.1 %, egg albumin 10 %, and 5-hydroxytryptamine 0.05 %. The volume of the paw was determined as described by KOFF & MØLLER NIELSEN (1958) before d 1½ hour after injection of the phlogistic. The test compound was injected 30 minutes before the phlogistic. The oedema-inhibiting effect was expressed as a percentage of the oedema in untreated controls (n = 10).

b) Thermoedema in rats (SPECTOR & WILLOUGHBY 1959). Anaesthetized rats were depilated on the bdomen. A circular area (diameter 2.0 cm) of the naked skin was exposed t heat injury by contact for 30 seconds with a copper plate, heated to 56 This resulted in the development of local oedema reaching a maximum in 2 hours. The rats were then killed with chloroform and skinned. The oedematous area was punched out, weighed, dried t 60 C and reweighed The weight difference is measure of the intensity of oedema When 0.4 ml per 100 g body weight f 1 % solution of trypan blue was injected L 2 hours before exposure to heat, the increase in capillary permeability caused by heat injury should result n extravasation of the dye in the oedematous area. The intensity of bluing was scored (0 to 4) The test compou d was injected 30 minutes before the exposure to heat. The inh biting effect of the compound was calculated by comparing the intensities of oedema and bluing in test nimals with those of untreated controls (n = 10).

c) Felt pellet test. This is a mod fication of the conventional cotton pellet test. Sterilized discs (diameter 4 mm), which had been punched out of a sheet of felt 5 mm thick were used

to stimulate granuloma formation Two discs were placed under sterile conditions subcutaneously the back of each rat. Thirteen days later the rat were killed, and the pellets with the granulatio tissue were removed, dried and weighed. Experimental animals were treated daily dur g the entire test period with the test compound. Inhibition of granulation in treated animals was calculated as percentage on the basis of the granulation in untreated controls (= 10).

d) Gran loma pouch test. Rats were anaesthetized with mebumal-Na and shaved on the back. Under aseptic conditions 20 ml of air were injected subcutaneously to form a subcutaneous pocket. Into this pocket was injected 0.5 ml of a 1 % sterile solution of croton il in peanut oil. The experimental animals were treated i.p. or p o. daily during the test period. The inhibition of exudation was calculated as percentage of the exudate in untreated controls (= 10). After 7 days the animals were killed, the pockets opened and the quantities of exudate measured.

Acute toxicity to mice and rats

Groups of 5 to 10 mice or rats were given increasing doses of meprotixol. The animals were placed in single cages at 22 24 and observed over a period of 96 hours. The LD50 was determined on the lethality t 24 hours by the method of MILLER & TAINTER (1944).

Results

Motility

Meprotixol reduces the spontaneous motor activity of mice, but its potency is negligible in comparison with those of neuroleptics such as chlorpromazine and chlorprothixene. Codeine is even weaker

Table 1

Reduction of spontaneous motor activity in mice. DR50 is the dose that reduces the activity to 50 % that of control mice. (n = 12).

Compounds	DR50 mg/kg i.p. (15 min)
Codeine.	15.0
Chlorp omazine	2.4
Clopenthixol.	0.46
Chlorprothixene.	0 4
Meprotixol	9.5

Potentiation of barbiturate anaesthesia

a) Although meprotixol, when given alone, is unable to induce anaesthesia in mice, it does prolong the anaesthesia induced by barbiturates.

Table 2

Potentiatio of enhexymal-N anaesthesia in mice. Half an hour after pretreatment, as indicated, 50 mg/kg enhexymal Na were given intravenously The potentiating fact r is defined as $\frac{\text{sleeping time, test animals}}{\text{sleeping time, control animals}}$ (n = 10).

Compound	Dose mg/kg i.p.	Potentiating fact r
Meprotixol	2.5	1 6
	25	>11
Codeine	2.5	0.6
	25	>3
Chlorpromazine	2.5	5
	25	26
Clopenthixol.	2.5	4
	25	18
Chlorprothixene	2.5	13
	25	>43

It may be seen from table 2 that meprotixol prolongs the anaesthesia of enhexymal, but this effect is much weaker than that of the neuro leptics, chlorprothixene in particular Codeine, on the other hand, shows some antagonism against barbiturate anaesthesia at the low dose level, whereas 25 mg show a weak potentiating effect.

b) The potentiating effect of meprotixol is related to dose, as can be seen from table 3

Table 3

Sleeping time of mice pretreated with increasing doses of meprotixol, i.p., 30 minutes before intra venous injectio f 50 mg/kg enhexymal-Na. (n = 10)

	sleepi g time, minutes
Control	3.3
Meprotixol 3.125 mg/kg	~10
6.25	~13
– 12.5	>19
– 25 –	>26

Table 4

Duration of barbiturate-potentiating effect of meprotixol in mice. Pretreatment with meprotixol, 25 mg/kg i.p. at various intervals before i. injection of 50 mg/kg enhexymal-N (n = 10).

	sleeping time, minutes
Control	3.3
Immediately after	~12.5
15 minutes	~23.3
30 minutes	~25.6
60 minutes	~15.6
120 minutes	6.2

c) From table 4 it appears that the effect of meprotixol (25 mg/kg i.p.) sets in rapidly reaches a maximum within about 30 minutes and lasts for somewhat more than 2 hours.

d) When meprotixol is injected intravenously into mice just awakening from enhexymal anaesthesia, they again revert to anaesthesia. As meprotixol itself has no anaesthetic effect, this phenomenon may be explained as a potentiation of the effect of a subanaesthetic concentration of the barbiturate on the central nervous system. It also means that the potentiation demonstrated under a, b and c is not due to an inhibitory action of meprotixol on the enzyme systems responsible for the breakdown of the barbiturate Table 5 shows the results of experiments with meprotixol.

Table 5

Potentiation f enhexymal-N anaesthesia in mice. Enhexymal-Na, 50 mg/kg, was given i.v and the sleeping time w recorded. At the time of wakeni g intravenous injections of various doses of meprotixol were given, nd the 2nd sleeping time was recorded. (= 10).

		mg/kg i.v	sleeping time minutes
I	Enhexymal	50	4 6
	+ meprotixol	2.5	>11
II	Enhexymal	50	5.4
	+ meprotixol	5	>1
III	Enhexymal	50	6.4
	+ meprotixol	10	>26

Table 6

Potentiation of thiomebumal Na anaesthesia in mice. Half an hour after pretreatment with increasing doses of meprotixol i.p. a 0.25 % solution of thiomebumal-Na was injected intravenously in divided doses of 0.05 ml every 10 seconds until anaesthesia ensued. Anaesthetic dose (ml) and sleeping time were recorded. (n = 10).

Pretreatment	mg/kg i.p.	ml 0.25 % thiomebumal-Na	sleeping time, minutes
None		0.30	1.5
Meprotixol	10	0.21	4.2
–	20	0.16	>12.7
–	40	0.11	>24.0

e) Meprotixol in suitable doses reduces the quantity of barbiturate required to secure anaesthesia. This is demonstrated in table 6, which gives the results of experiments in which the anaesthetic dose of thiomebumal Na is "titrated" by fractional intravenous injection until anaesthesia is observed. It can be seen that not only does the anaesthetic dose of thiomebumal decrease with increasing dose of meprotixol, but also that the anaesthesia is considerably prolonged. It should be mentioned that the effect of meprotixol, however clear is decidedly weaker than the similar action of neuroleptics Thus chlorprothixene yields similar potentiating action at much smaller doses, i e. 0 125 mg/kg

Potentiation of ethanol anaesthesia in mice

Table 7 shows that meprotixol in doses from 10 to 40 mg/kg caused significant reduction in the dose of alcohol required for anaesthesia and

Table 7

Potentiation of ethanol anaesthesia in mice. Half an hour after pretreatment with increasing doses of meprotixol i.p. 20 % solution of ethanol was injected intravenously at a rate of 0 05 ml every 10 seconds until anaesthesia was observed. Anaesthetic dose (ml) and sleeping time were recorded.

Pretreatment	mg/kg i p.	ml 20 % ethanol i. t anaesthesia	sleeping time minutes
None		0.30	0 9
Meprotixol	5	0.24	1.2
–	10	0 18	>16.8
–	20	0 15	>18.2
–	40	0.09	>24.2

Table 8

Effect o body temperature of rats. Maximal fall in body temperature fter i p. injection of a standard dose of 5 mg/kg.

Compound	mg/kg i.p.	Max. fall in body temperature, °C
Meprotixol	5	0.9
Chlorpromazine	5	3 6
Chlorprothixene	5	4.6
Clopenthix l	5	2.5

that the sleeping time was considerably prolonged The potency of meprotixol, however is weak in comparison with that of neuroleptica. Chlorprothixene, for instance, will show a similar effect in doses of the order of 1 or 2 mg/kg.

Effect on body temperature of rats

The temperature reducing effect of meprotixol is negligible in comparison with that of neuroleptics (table 8).

Anticataleptic effect vertical rod test on mice

Meprotixol showed slight anticataleptic effect against the cataleptic reaction induced by chlorprothixene 2.5 mg/kg s.c. In mice pretreated with 10 mg/kg meprotixol s.c. the average score for cataleptic reaction was significantly reduced (table 9)

Table 9

Antagonism gainst cataleptic reaction in mice Pretreatment with meprotixol, 10 mg/kg s.c 30 minutes bef re the cataleptogenic gent (chlorprothixene 2.5 mg/kg s.c.). The cataleptic reaction was scored as indicated under methods.

Pretreatment	mg/kg		Average score cataleptic react.	Number of animals score ≤ 3	score ≥ 10
Control	none	10	11 4	0	9
Meprotixol	10	10	5 4	3¹)	1²)

) 90 < P < 95   ²) P > 99.95.

Table 10

Effect on $\overline{LD100}$ of various toxic agents. Meprotixol, 40 mg/kg i.p. was given 30 minutes before i injection of toxic agent in divided doses (0.05 ml every 10 seconds). The volume of the toxic solution was recorded. Ratio is $\frac{\overline{LD100} \text{ test (ml)}}{\overline{LD100} \text{ control (ml)}}$ Ratios >1 indicate protection.

Toxic agent	mg/ml	$\frac{\overline{LD100} \text{ test}}{\overline{LD100} \text{ contr l}}$	ratio
Nicotine bit.	1	0.24/0.09	2.67 P[1]) > 99.95
Strychnine nitr	0.1	0.22/0.18	1.22 99.9 < P < 99.95
Harmine, HCl	10	0.23/0.20	1 15 99.5 < P < 99 9
Acetylcholine, HCl	10	0.13/0.12	1.08 70 < P < 80
Pentetrazol	10	0.14/0.12	1 17 P ~ 90
Mescaline, sulph	50	0.19/0.19	1 00 60 < P < 70
Picrotoxin	5	0.30/0.23	1.31 P > 99.95

1) P values are based on the t-test fo $\overline{LD100}$-values of test and control.

Antireserpine effect on mice

Meprotixol in doses as high as 32 mg/kg i p had no antagonistic effect against reserpine ptosis. This is in contrast to the true thymoleptics, such as amitriptyline, which showed significant antagonism at doses of 1 mg/kg.

Influence on $\overline{LD100}$ of convulsive and other toxic agents

From table 10 it appears that meprotixol 40 mg/kg i p given 30 minutes before the toxic agent rendered protection against convulsions and death induced by nicotine, harmine, picrotoxin or strychnine, whereas the protective effect against mescaline, pentetrazol or acetylcholine was insignificant or nonexistent. Facilitation of convulsions was not demonstrated.

Protection against electroconvulsions in mice

Meprotixol showed marked protection against the tonic component of maximal electroconvulsive seizures in mice. In table 11 the protective dose PDMES50 for meprotixol is determined together with that of a number of neuroleptics and of codeine. It is evident that meprotixol has a stronger anticonvulsive effect than most of the other substances.

Table 11

Protection against maximal electroconvulsive seizures in mice. Eye-electrodes, 9.5 mA 0.2 sec.

Compound	PDMES 50 mg/kg i.p.
Meprotixol	9 6
Chlorprothixene	11.5
Chlorpromazine	28.0
Clopenthixol	32.0
Perphenazine	38.0
Codeine	40.0

Effect on righting reflex in rats

Meprotixol had no inhibitory effect on the righting reflex of rats even at toxic dose levels.

Holding reflex rotating drum-test on mice

The ED50 of meprotixol was 11 2 mg/kg i.p

Balance test rotating rod on rats

The ED50 of meprotixol was 20 0 mg/kg i.p

Antitussive effect on cat

Meprotixol inhibited the cough response provoked by stimulation of the superior laryngeal nerve in anaesthetized cats. The compound was compared with codeine by both intravenous and oral administration. Table 12 shows the individual results of comparative experiments with meprotixol and codeine It appears that meprotixol is about 4 times as potent as codeine.

A portion of the kymographic recording of an experiment in which the cough responses were temporarily depressed after i.v administration of meprotixol, 1 0 mg/kg, is shown in fig. 2.

Effect on respiration

In experiments on rabbits, anaesthetized with urethane, meprotixol at doses of 0,06 to 1 mg/kg i v had no effect on respiratory frequency or volume. Doses from 0.25 to 1 mg/kg caused a transient increase in

Table 12

Antitussive effect anaesthetized cat. Superior laryngeal nerve stimulation.

Compound	mg/kg	Route	Latent period minutes	Duration of antitussive effect, minutes
Codeine	1	i.v.	<5	15
–	1	–	–	40
–	1.25	–	–	20–25
–	1.25	–	–	40
–	2	–	–	40
–	2	–	–	20–25
–		–	–	45–60
–	2	–	–	35
–	2	–	–	15
–	2	–	–	20
–	2	–	–	70
–	2	–	–	50
–	2	–	–	50
–	4	–	–	30
–	5	–	–	40
–	5	p.o.	10–20	10–20
–	5		40–45	25
–	5	–	25	35
–	10	–	10–15	>65
–	10	–	10–15	25–30
Meprotixol	0.25	i.	<5	35
–	0.25	–	<5	35–45
–	0.25	–		0
–	0.25	–		0
–	0.25	–	<5	15
	0.5	–	<5	30–40
	0.5	–	<5	25–35
–	0.5	–	<5	45
–	0.5		<5	30
–	0.5	–	<5	15
–	0.5	–	<5	25–30
–	0.5	–	<5	15
–	1.0	–	<5	25
–	1.0	–	<5	25–35
–	1.0		<5	>60
–	2.0	–	<5	>120
–	1.0	p.o.	10	15
–	1.0	–	25	15
–	1.0	–	30–40	30–40
–	2.0	–	20–30	20–30
–	2.0	–	10	>40

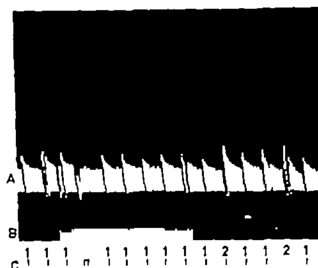


Fig. 2. Antitussive effect, anaesthetized cat, superior laryngeal nerve stimulation.

A. Respiration and cough response.
B. Minutes.
C. Signal 1 = stimulatio laryngeal nerve
2 = double stimulation laryngeal nerve
□ = meprotixol 1.0 mg/kg i.

Duration of inhibition of cough response pprox. 50 minutes.

amplitude. In cats, anaesthetized with chloralose-urethane 0.25 to 0 5 mg/kg, meprotixol i v caused no changes in respiration A dose of 1 mg/kg produced an increase in respiratory volume of 25 / 2 mg/kg increased the amplitude, the respiratory volume being slightly reduced (20%) The frequency was initially increased for one minute before a slight reduction. A dose of 8 mg/kg i.v caused respiratory arrest and 16 mg/kg caused cardiac arrest

Effect on potential waves recorded from the phrenic nerve

Intravenous injection of meprotixol 2 mg/kg, had no effect on the height, length or frequency of *the impulse waves*

On the other hand 2 mg/kg codeine phosphate i.v caused a shortening of the impulse waves and a decrease in frequency with normalization within 15 minutes.

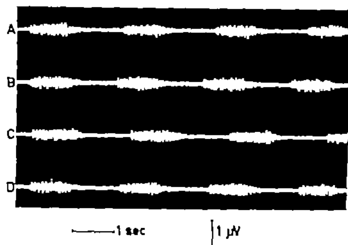


Fig. 3 Impulse waves recorded from the phrenic nerve in the cat (chloralose-urethane anaesthesia)
A before injection B, C and D 1 15 and 30 minutes, respectively after intravenous injection of 2 mg/kg meprotixol.

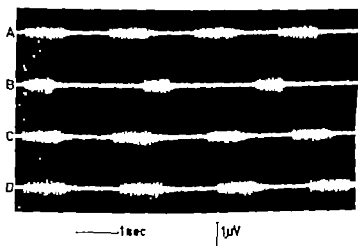


Fig. 4 Impulse waves recorded from the phrenic nerve in the cat (chloralose-urethan anaesthesia)
A. before injection B, C and D 3 15 and 30 minutes, respectively after intravenous injection of 2 mg/kg codeine phosphate.

Effect on blood pressure

In anaesthetized rats (urethane) meprotixol, 2 mg/kg i.v had a moderate hypotensive effect of about 15 / of the initial level In contrast to neuroleptics, the pressor effect of adrenaline was not affected by this

dose. In anaesthetized cats (chloralose-urethane) the effect of meprotixol was studied in doses ranging from 0.078 to 10 mg/kg i.v. Doses under 1 mg/kg had no effect on blood pressure. When large doses (2.5 to 10 mg/kg) were injected, an increasing initial depressor effect, from 25 to 70 % of the initial level, led to a long lasting reduction in blood pressure level of about 10 to 15 %.

Electrocardiographic studies

In chloralose urethane anaesthetized cats no ECG changes were seen after intravenous injection of 0.25 to 1 mg/kg, but 2 to 4 mg/kg i.v. delayed the atrioventricular impulse transmission (PQ segment) and caused a widening of the ventricular complex (QRS). A dose of 8 mg/kg caused ventricular fluttering.

In rabbits lightly anaesthetized with N_2O/O_2 (4/1) one mg/kg i.v. was without effect. One minute after i.v. injection of 2.5 mg/kg a slight broadening of the QRS-complex occurred, indicating a partial branch block. Further, the atrioventricular impulse transmission (PQ segment) was delayed. The ECG was normalized within 15 minutes. A dose of 5 mg/kg i.v. caused ventricular tachycardia, which subsided after 30 minutes.

Autonomic effects

In anaesthetized rabbits (urethane) meprotixol, 0.125 to 4 mg/kg i.v. showed no effect on the tone or motility of jejunum or uterus in situ.

Effect on the function of the nictitating membrane

In experiments on anaesthetized cats (chloralose-urethane) meprotixol was injected intravenously at doses ranging from 0.078 to 5 mg/kg. Doses of 1.25 or more caused an equal reduction of the response to both pre ganglionic and postganglionic stimulation of the cervical sympathetic trunc. Similarly, the response to noradrenaline was increasingly reduced by meprotixol at doses from 0.078 to 5 mg/kg i.v. Thus the inhibitory effect of meprotixol on the function of the nictitating membrane is considered to be localized at the effector organ and not at the superior cervical ganglion.

Adrenaline, noradrenaline and 5-hydroxytryptamine antagonism

In blood pressure experiments on pithed rats meprotixol showed weak antagonistic effect against the pressor responses of adrenaline and nor-

adrenaline The ED50 could not be determined within the tolerated dose range (up to 10 mg/kg i.v.) The antagonism against 5-hydroxytryptamine compared to that of neuroleptics, was also relatively weak, the ED50 being 1 8 mg/kg i.v.

Acetylcholine antagonism

Meprotixol had no significant antagonistic effect against acetylcholine spasms in the isolated ileum preparation from guinea pigs.

Effect on pupil diameter

Meprotixol had a weak mydriatic effect on mice. An increase in pupil diameter of 100 / was obtained with 26 mg/kg i.p.

Broncholytic effect

Meprotixol showed a potent *bronchospasmolytic* effect against 5-hydroxytryptamine spasms in guinea pigs. Thus 0 08 mg/kg meprotixol i.v. inhibited the spasms by 50 / In contrast, the spasmolytic effect against acetylcholine spasms was relatively weak, 50 / inhibition requiring 7 4 mg/kg i.v.

Antinociceptive activity

Meprotixol showed weak analgesic effect in all tests performed. Table 13 compares the ED50 s of meprotixol, morphine and codeine.

It should be mentioned that meprotixol causes a slight increase in the analgesic effect of morphine. When used with a subanalgesic dose of morphine (5 mg/kg s.c.) meprotixol, 27.5 mg/kg, increases the reaction time to above 30 seconds in 50 / of test animals.

Table 13

Antinociceptive activity in mice.

Compound	ED50 (mg/kg), antinociceptive test methods			
	Tail pinch	Phenylquinone	Hot plate	Tail stimulation
Morphine	1 p	1.0 s.c.	14 s.c.	4 i.p.
Codeine	50 p.	23 s.c.	35 s.c.	18 i.p.
Meprotixol	85 s.c.	5 p	145 s.c.	27 i.p.

Table 14

Inhibition of oedema produced by various phlogistics in the paws of anaesthetized rats (urethane).

Compound	mg/kg i.p.	% inhibition		
		dextra 0.1	albumen 10%	5-hydroxy-tryptamine 0.05 %
Meprotixol	25	55	66	59
–	50	51	82	80
Na-salicylate	400	26	43	24
Phenylbutazone	100	32	–	30
	200		36	–

Local anaesthetic effect

After intraconjunctival instillation, a local anaesthetic effect of meprotixol was observed The ED50 was 0.32% For comparison the ED50 of lidocaine was 1.38% of cocaine 0 77% and of tetracaine 0 2%

Antiphlogistic effect

a) Rat paw oedema Table 14 shows that meprotixol in smaller doses possessed far better inhibitory effect against all three types of oedema than did sodium salicylate and phenylbutazone.

b) Thermoedema in rats With a dose of 25 mg/kg i p of meprotixol oedema was inhibited by 19% and dye-diffusion by 18% when recorded 2 hours after heat injury

c) Felt pellet test In this test meprotixol showed only slight inhibition of granuloma formation The results found were 25 mg/kg i.p 5% inhibition, 100 mg/kg i p 7% inhibition and 100 mg/kg orally 14% inhibition.

d) Granuloma pouch test meprotixol, 12.5 and 25 mg/kg i p daily for 6 days reduced exudation in the pouch by 35 and 43% respectively With an oral dose of 50 mg/kg daily inhibition was not observed

Acute toxicity

The results of acute toxicity tests on mice and rats are shown in table 15

Table 15

LD50 f r meprotl ol in mice and rats after intravenous, intraperitoneal or oral dminbtration.

	LD50, mg/kg ± S.E.M			
	mice	n	rats	n
Intra enous	60 ± 4	20	33 ± 3	15
Intraperito eal	125 ± 11	50	110 ± 13	20
Oral	420 ± 47	40	430 ± 57	20

Summary

Meprotixol, a thiaxanthene derivative, has been examined pharma cologically It appears that the compound combines a low central depressant effect with an antitussive effect about 4 times greater than that of codeine. In contrast to codeine, meprotixol showed no respiratory depressive effect on the contrary a certain stimulatory effect was observed Meprotixol inhibits the tonic extensor component of the maximal electro-shock seizure. Moreover this compound has a marked inhibitory effect against various forms of oedema and exudation in the granuloma pouch, though the inhibition of granuloma formation was only slight.

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From the Department of Pharmacology Royal Veterinary College,
and the Department of Biochemistry Royal Institute of Technology
Stockholm Sweden

Autoradiographic Studies on ^{35}S-Thiamine Distribution in Mice

By

L. Hammarström, H. Neujahr and S. Ullberg

(Received August 26, 1965)

Most of the available information on thiamine distribution in animals is based on results of chemical or microbiological assays of extracts from organ homogenates (SPECTOR 1956 LONG 1961). Such results are necessarily fragmentary and give no information on the distribution within the tissues.

COHEN *et al* (1962) have, using autoradiography compared the distribution of ^{35}S-thiamine with that of the fat soluble derivative dithiopropylthiamine. They reported a high accumulation of thiamine in the liver and kidney and a lower concentration in the myocardium, brown fat and brain

In the animal body thiamine is converted to thiamine pyrophosphate, which is the most abundant form of thiamine. In nervous tissue thiamine monophosphate and thiamine triphosphate have been found besides thiamine diphosphate, and several investigators report that free thiamine is liberated on stimulation (VON MURALT 1962). Small amounts of thiamine triphosphate have also been isolated from liver and brain (KIESSLING 1961)

Thiamine pyrophosphate is the coenzyme by whose action the end products of anaerobic metabolism of glucose and aliphatic acids are made available for complete oxidation by aerobic systems. This function of thiamine in glucose metabolism justified us in comparing the autoradiographic distribution pattern of thiamine with that of glucose which has recently been studied in our laboratory (ULLBERG & KORANSKY unpublished results)

In spite of the comparatively large amount of information on thiamine function in enzymic *in vitro* systems, the relation between such function

and clinical symptoms in thiamine deficiency is still quite obscure. It was hoped that our whole body autoradiographic investigation might contribute to the elucidation of such problems.

Methods

^{35}S-labelled thiamine was obtained from Hoffmann-La Roche & Co Basle, Switzerland. The specific activity of the preparation t the time of injection was 75 µC/mg. The substance was dissolved in physiological saline, and 0.2 ml was i jected into the tail veins f mice. Th individual dose was 0 26 mg thiamine, corresponding t bout 19 µC.

Thirteen whit mice, 6 ad lt males (verage weight 25 g) and 7 pregnant females (average weight 38 g) were used. The intervals between i jection and sacrifice were for the males and six of the female mice 5 min., 20 min., 1 hour 4 hours, 24 hours and 4 days. The female mice were killed two d ys before expected parturition. The foetuses from the additional female were removed 24 hours after the intravenous injection of the mother and treated separately

The animals were killed by immersion in hexane cooled to −75 with solid carbon dioxide. Sagittal sections 20 µ thick were cut at various levels thro gh the whole animals at −10 The sections were dried t the same temperature and autoradiographic exposure was made by apposition against Structurix X-ray film (Gevaert). The exposure time was –4 weeks. The autoradiographic method has been previously described in detail (ULLBERG 1954 & 1958).

Results

The distribution of the radioactivity after intravenous injection of ^{35}S-thiamine was characterized by a gradual decrease in blood concentration which was completed after 4 hours, and an accumulation in the tissues, especially in the liver the renal cortex, the corpora lutea, the brown fat and the Harderian gland. A rapid but transient accumulation was also observed in the thyroid gland, in the walls of the aorta and the arteries and in the cartilage.

The distribution in various tissues at different time intervals will be described in detail below

Circulatory system

The radioactivity gradually left the blood after the intravenous injection after 4 hours no activity could be observed in the circulating blood

There was a slow increase of radioactivity in the myocardium the accumulation was high 4 days after the injection. The walls of the aorta and of the arteries showed an initial high concentration which then gradually decreased and reached a low level after 24 hours.

A slow and moderate uptake was observed in the bone marrow the spleen, the thymus and the lymph nodes.

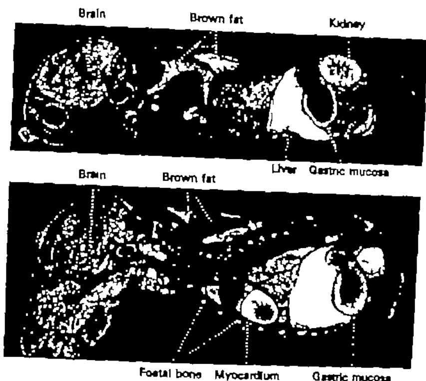


Fig. 1 Autoradiogram of two full term mouse foetuses 24 hours after an intravenous injection of 35S-thiamine into the mother White areas correspond to high concentratio of radioactivity Note the high concentrations in brown f t, myocardium, liver gastric mucosa and renal cortex. Note also the uptake in developi g bone.

Nervous system

In the brain radioactivity accumulated slowly but after 2 days the concentration was fairly high. A moderate concentration was seen in the *trigeminus* nerve as soon as 5 minutes after the injection and this concentration was still unchanged after 4 days. Five minutes after the injection a higher uptake was observed also in the spinal ganglia and in the semilunar ganglion. The accumulation in these ganglia proceeded more rapidly than in the brain, but reached the same final level

Digestive system

In the oral and oesophageal mucosa no observable amounts of labelled substance accumulated In the gastric mucosa the concentration of radioactivity was low after 5 minutes, but it increased to a high level after one hour and was still high after 4 days. The activity of the muscular layer of the gastrointestinal wall was low

A rapid high and long-lasting accumulation was observed in the submaxillary salivary glands.

The liver had a high level of radioactivity throughout the investigation period

A moderate concentration was seen in the pancreas after short intervals. After one hour the activity of the pancreas had increased to about the same level as that of the liver but later on it decreased more rapidly than the liver activity

Respiratory system

A high concentration of radioactivity was observed in the nasal cavity The tracheal cartilage also showed a strong accumulation of radioactivity after 5 and 20 minutes, but the concentration decreased and was low after one hour

After 5 minutes the activity in the lungs was equal to that in the blood, but the radioactivity of the blood disappeared, whereas that of the lungs remained after 4 days. The highest concentration in the lungs was seen in the bronchial walls.

Urinary system.

The kidney showed a high concentration of ^{35}S-activity during the whole period Initially the concentration was high in both the cortex and the medulla, but after longer intervals only the cortical tissue, especially the inner cortex, showed high activity There was a high concentration of radioactivity in the ureters and in the urinary bladder after short survival periods.

Endocrine system

The pituitary showed a higher concentration of radioactivity than the brain proper Within the pituitary the highest concentration seemed to be confined to the posterior lobe

In the thyroid accumulation was observed mainly concentrated in small spots. The accumulation had disappeared after 24 hours

There was a slow accumulation of radioactivity in the adrenals. The concentration was slightly higher in the cortex than in the medulla.

The concentration in the islets of Langerhans was low at all the intervals studied.

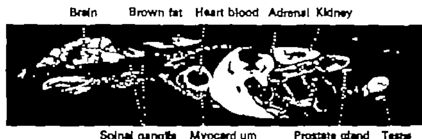


Fig. 2. Autoradiogram of an adult male mouse 4 days after an intravenous injection of ^{35}S-thiamine. Note the high concentrations in brown fat, myocardium, liver, kidney and prostate gland. The concentrations are comparatively high in brain, spinal ganglia and testis.

Reproductive system

The testes showed fairly high concentration of radioactivity in the interstitium. An accumulation was also observed in the epididymis and in the walls of the ductus deferens. The concentration was high in the prostate gland.

In the ovaries a high uptake was observed in the walls of some large follicles 5 minutes after the injection the follicular fluid also showed accumulation of radioactivity After one hour the radioactivity in the corpora lutea had increased considerably to about the same high concentration as that of the liver and also remained high throughout the investigation

The placenta showed about the same concentration as the blood, but the activity in the placenta persisted, whereas that in the blood decreased The concentration of radioactivity in the uterine wall was moderate throughout the investigation period

Mammary glands

In the mammary glands a moderate concentration was observed throughout the investigation period.

Skeletal tissues and muscles

No uptake was observed in compact bone. Cartilage showed a strong accumulation initially but this had largely disappeared after 4 hours. In the permanently growing incisors the activity was especially concentrated in the enamel. The skeletal muscles showed a fairly low concentration throughout the investigation



Fig. 3 Autoradiogram of pregnant mouse 20 mi utes after an intravenous injection of 14C 1-glucose. Note the high concentrations in brain, Harder s gland, brown fat, myocardium, liver nd gastric mucosa. (From ULLBERG & KORANSKY unpublished results.)

Brown fat

The concentration in brown fat increased during the first hour after injection to a high level, which persisted throughout the investigation period.

Harderian gland

The uptake in the Harderian gland was similar to that in brown fat.

Serous cavities

A fairly high activity was observed in the serous cavities during the first hour The radioactivity was concentrated to minute spots, possibly representing mast cells.

Foetuses

The radioactivity gradually appeared in the foetuses, reaching about the same concentration as in the mother after 4 days. A selective accumulation was seen in the gastric mucosa, but not in the intestinal mucosa. A high accumulation was also seen in the foetal liver kidneys, brown fat and cartilage. In the foetal central nervous system the concentration gradually reached a level similar to that in the mother

Discussion

An essential question when interpreting the autoradiograms is to what extent the 35S-radioactivity represents thiamine and its phosphate derivatives or degradation products Previous studies on thiamine metabolism

have mainly been performed on urine and faeces (McCARTHY *et al* 1954 IACONO & JOHNSON 1957 VERRETT & CERECEDO 1958) NEAL & PEARSON (1964) using ^{14}C thiamine found that 48–51 / of the radioactivity recovered in the urine of long term injected rats represented nondegraded thiamine Those of the published values based on tissue analyses indicate that degradation of thiamine may occur mainly in association with the disappearance of the vitamin from the tissues. Thus WISS & BRUBACHER (1962) found thiamine diphosphate to be the only radioactive component in the liver of thiamine-depleted rats to which small doses of ^{35}S-thiamine had been administered COHEN *et al* (1962) got a similar result with larger doses of ^{35}S-thiamine.

The distribution of ^{35}S-labelled Heminevrin ® the thiazole moiety of the thiamine molecule, has been investigated by ALLGÉN *et al* 1963 The distribution was highly uniform in most tissues except for an accumulation in the liver Thus the distribution of the thiazole moiety a compound that seems to appear in animal tissues only as part of thiamine, shows little resemblance to the diversified pattern obtained after injection of ^{35}S-thiamine

A comparison of the autoradiographic distribution pattern after injection of ^{35}S-thiamine with that after injection of ^{14}C-6-glucose reveals several similarities (ULLBERG & KORANSKY unpublished results) With both substances a selective accumulation was obtained in the myocardium, arterial walls, gastric mucosa, brown fat and Harderian gland. There was also a rapid but transient localization of both in the cartilage. Both substances showed a low concentration in the adrenal medulla and in skeletal and intestinal muscles In the nervous system the autoradiograms of both ^{35}S-thiamine and ^{14}C glucose showed accumulation in both CNS and peripheral nerves. The uptake was higher in the grey matter than in the white and higher in the ganglia than in peripheral nerves. However ^{14}C-glucose was taken up more rapidly and to a higher extent than ^{35}S-thiamine.

Changes in the cardiovascular system are among the most prominent symptoms of thiamine deficiency Cardiac arrhythmias appear in early deficiency and enlargement of the *heart* and low blood pressure have been reported in severe beriberi (SEBRELL Jr 1962). The high concentration in the myocardium may indicate a direct action of thiamine on the heart muscle. Also the *arterial walls* showed a high uptake, and these sites of accumulation may be of importance for the normal function of the cardiovascular system. Thiamine has been shown to increase capillary resistance in guinea pigs (PARROT & COTEREAU 1949)

Thyroid tissue has been found to be capable of producing $^{14}CO_2$ from labelled glucose and also from ^{14}C pyruvate (FIELD *et al* 1961) The

accumulation of thiamine in the thyroid may be due to its function as coenzyme in these processes. Therapeutic doses in man may produce symptoms similar to those of thyroid hyperactivity (MILLS 1941)

^{35}S-sulphate is known to accumulate in *cartilage* but the transient accumulation observed in our investigation can hardly represent this degradation product of ^{35}S-thiamine, since sulphate shows a slowly in creasing and long lasting accumulation in cartilage.

Summary

The distribution of ^{35}S-thiamine in male and pregnant female mice after intravenous injection has been investigated by whole body autoradiography There was a slow decrease in blood concentration The liver renal cortex, corpora lutea, brown fat and Harderian gland showed a high and persistent accumulation A rapid but transient accumulation was observed in the thyroid gland, the aortic and arterial walls and the car tilage. The central nervous system showed a slow uptake, but a compara tively high final concentration. The distribution pattern after injection of ^{35}S-thiamine exhibits close similarities to the previously studied distribution of ^{14}C-glucose.

Acknowledgements

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From the Department of Pharmacology University of Göteborg, Göteborg, Sweden.

Anaerobic Glycolysis in Isolated Diaphragm from Thyroxine Treated and Untreated Rats

By

Nils Svedmyr

(Received September 24, 1965)

In earlier experiments it was found that the stimulation by adrenaline of lactic acid production in the rat diaphragm was potentiated by administration of thyroxine (SVEDMYR 1965 a). In different series of experiments attempts have been made to localize the point of attack of this effect. Thyroxine did not affect the glycogenolytic or phosphorylase-activating effects of adrenaline (SVEDMYR 1965 a & b) or its effect on the concentrations of hexose phosphates (SVEDMYR, unpublished results) According to GOLDSTEIN (1952) however anaerobic glycolysis is increased in the rat diaphragm after thyroxine treatment. BARGONI *et al* (1961) found that thyroxine increased the content of enolase and lactic acid dehydrogenase in the rat liver NIKKILA & PITKÄNEN (1959) demonstrated an increased content of phosphoglyceric acid kinase in thyrotoxic human liver Finally HARARY (1957 & 1958) found that the enzymatic activity of acylphosphatase was increased in rat liver and muscle after thyroxine administration. This enzyme hydrolyses, among other substances, 1 3-diphosphoglyceric acid to 3-phosphoglyceric acid and may therefore conceivably act as an "uncoupler" of the glycolysis from the phosphorylation If the rate of glycolysis is limited by phosphate acceptors (ADP) or inorganic phosphate or both, an increased acylphosphate activity could stimulate this rate.

A number of observations reported in the litterature thus indicated that thyroxine treatment could potentiate later links in the glycolytic chain Such an effect of thyroxine could eventually explain its potentiating effect on the stimulation of lactic acid production by adrenaline. I have therefore compared the rates of anaerobic glycolysis in diaphragms from

thyroxine treated and untreated animals. In an attempt to find out whether there was any 'uncoupling' of glycolysis and phosphorylation, the contents of adenosine triphosphate (ATP) and creatine phosphate (CrP) were also determined.

Methods

The animals in the thyroxine treated group were injected subcutaneously with thyroxine in a dose of 100 μg/animal/day for 7 days. The basal metabolism increased by approximately 30 %. The animals weighed between 200 and 250 g. In order that the muscle should have as high an initial glycogen content as possible, the animals were not fasted before the experiments.

Of the rats 4 were killed by a blow on the neck. The diaphragms were dissected out and incubated in 15 ml ice cold Krebs Henseleit bicarbonate buffer containing 0.2 % glucose. The solution was gassed with 95 % O_2 + 5 % CO_2. In order that different groups of diaphragm sections should have as similar compositions as possible, the muscular part of each diaphragm was divided sagitally into four equally sized sections 1 left lateral, 1 left medial, 1 right medial and 1 right lateral. From each original diaphragm one section was then taken and pooled with sections from the other diaphragms so that 4 groups were obtained, each consisting of 1 left lateral, 1 left medial, 1 right medial and 1 right lateral section, but with the sections from different animals. When these groups had been incubated for 30 minutes in ice cold bicarbonate buffer one group (the control group) was frozen with freon 11 containing dry ice. The other groups were incubated in 15 ml bicarbonate buffer containing 0.2 % glucose, at 37° The solution had been gassed for the previous 30 minutes with a mixture of 95 % N_2 + 5 % CO_2. After 15, 30 and 60 minutes a group of diaphragm sections was taken for analysis, and 1 ml of the suspension solution was also analysed for lactic acid.

After freezing, the muscle preparation was weighed and homogenized in 8 volumes of ice cold 6 % perchloric acid. After centrifugation the perchloric acid was neutralized with potassium carbonate. The potassium perchlorate was centrifuged down, and the extract was analysed for ATP by the method of ADAM (1962) for CrP by that of LUNDHOLM & VAMOS (1965) and for lactic acid by that of LUNDHOLM, MOHME LUNDHOLM & VAMOS (1963).

The amount of lactic acid produced was determined from the quantity of lactic acid in the suspension solution and muscle at the end of the experiment less the lactic acid content of the muscle at the beginning.

Results

The extent of anaerobic glycolysis at different times and the contents of ATP and CrP are given in fig. 1 and table 1 Lactic acid production was greatest during the first 15 min in both treated and untreated animals. Lactic acid production during this period was almost identical in thyroxine treated and untreated animals. The changes in content of ATP and CrP were almost identical in the two groups during the first 15 min. period. After 30 and 60 min. respectively the rate of lactic acid production decreased in both untreated and thyroxine-treated animals. The reduction was greater however in the untreated animals, so that after

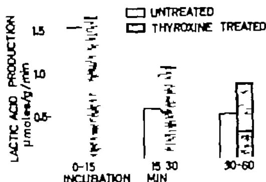


Fig. 1 The rate of lactic acid productio in µmoles/g/min. during different periods of incubation in diaphragms from ntreated and thyroxine treated rats.

Table 1

Effect f thyroxine treatment on spontaneous anaerobic glycolysis, ATP d CrP contents f isolated rat diaphragms after different times of incubation. Figures indicate means ± s.e.m.

	Untreated = 5	Thyroxine treated = 7	Difference betwee thyroxine treated and nt eated
Lactic acid production µmol/g			
0–15 min.	23 0 ± 1 83	25 1 ± 1.94	2.1 ± 2.67
0–30	31 8 ± 3.16	41.3 ± 2.33	9.6 ± 3.89 $P < 0.05$
0–60	47 0 ± 5 44	66.6 ± 2.49	19 6 ± 6.11 $P < 0.01$
ATP concentr tion µmol/g			
basal	3.33 ± 0 39	3 49 ± 0.18	0 16 ± 0.43
15 min.	1.20 ± 0.22	1..4 ± 0.10	0.04 ± 0.24
30	0 75 ± 0.22	1 01 ± 0.12	0.26 ± 0 26
60	0.61 ± 0.35	0 77 ± 0.13	0.16 ± 0.37
C P concent ation µmol/g			
basal	3 65 ± 0.51	4 50 ± 0.27	0.85 ± 0.57
15 mi	0.57 ± 0.13	0.47 ± 0.07	−0.10 ± 0.15
30	0.52 ± 0 16	0 43 ± 0.13	−0.09 ± 0 21
60	0.43 ± 0.09	0.28 ± 0.07	−0.15 ± 0.12

4*

30 min. and 60 min. the total lactic acid production was significantly greater in the thyroxinetreated than in the untreated animals The ATP content continued to fall in both groups It appeared somewhat higher however after both 30 and 60 minutes in the thyroxine treated than in the untreated animals, but the difference was not statistically significant

Discussion

The lactic acid production showed its maximal increase during the first 15 min in both untreated and thyroxine treated animals. No difference in the maximal rate of glycolysis was found between untreated and treated animals. The reduction in ATP production was also of the same magnitude in the two groups during the first 15 minute period. These results indicate that the administration of thyroxine did not affect the maximal rate of glycolysis and that ATP production from glycolytic processes was probably the same in untreated and thyroxine treated animals. There appeared to be no indication that any possible increase in enzyme concentrations was of importance for the maximal rate of glycolysis. Neither were any signs found of an "uncoupling" effect between glycolysis and phosphorylation as has been suggested by HARARY (1957 & 1958)

After 30 and 60 min lactic acid production was higher in thyroxine treated than in untreated animals this was because, as may be seen in fig. 1 the reduction in rate of glycolysis was smaller after thyroxine than in untreated animals. If the rate of glycolysis had only been determined after 60 min for example, this effect would have given the impression of potentiated glycolysis after thyroxine administration

The decrease in rate of glycolysis after 15 min. may be explained in various ways. The glycogen reserves of the muscle may possibly be used up after this period The reduction in the ATP content was probably due to the decreased glycolysis. Both the hexokinase and phosphofructokinase reactions utilize ATP however if the ATP content decreases sufficiently it is possible that these reactions may be rate-limiting in the glycolysis chain In fig. 2 the rates of lactic acid production during the periods 0–15 15–30 and 30–60 min. have been correlated with the mean ATP contents (the mean values of ATP at the beginning and the end of the period) As may be seen from the figure, the decreases in the rate of lactic acid production and the contents of ATP ran fairly parallel There was some difference between the curves for thyroxine treated and untreated animals. Thyroxine treated animals had a higher rate of lactic acid production than untreated animals with the same ATP content. This may indicate that the lactic acid production in the diaphragm was affected to a lesser degree by ATP reduction after thyroxine treatment than in untreated animals.

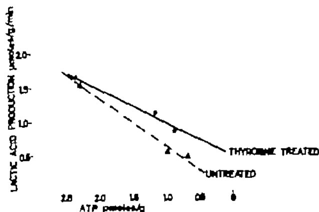


Fig. 2. The relationship between the rate f lactic acid productio and the mea ATP concentration in diaphragms from u treated and thyroxine-treated rats.

Summary

On incubation of isolated rat diaphragm in Krebs-Henseleit bicarbonate buffer with 0.2 / glucose, the anaerobic rate of glycolysis was highest during the first 15 minutes. No difference in the maximal rate of glycolysis was found between diaphragms from untreated and thyroxine-treated animals After 30 and 60 min incubation the rate of glycolysis decreased in both groups, but the decrease was considerably smaller in the thyroxine treated animals. During these periods thyroxine treatment appeared to potentiate the anaerobic glycolysis The decreases in the contents of ATP and CrP were similar in both groups, and there appeared to be no indications that thyroxine had "uncoupled" the glycolysis from the phosphory lation.

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From the Biological Department, the Pharmaceutical Institute, University of Oslo

The Release of Kinins in Human Plasma Substrates

By

Kjell Briseid, Olav Kjell Dyrud and Sidsel Flem Rimvik

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The significance of the results obtained by existing methods for determining human plasma kininogen is reduced by our incomplete knowledge of the kinin system of blood. Not only are the properties and the mode of functioning of the different kinin releasing and inactivating factors only partly known, but it has also not yet been settled whether or not there exist more than one kininogen. The differences in biological activities of the known kinins make it a matter of special interest, in the determination procedure, to know which kinins are set free by the different releasing enzymes employed. Information about the specificities and effects of such enzymes can be found in several papers, but the interpretation of the results is complicated by the fact that plasmas from different species have been used and that small differences in preparation procedures may affect both the properties of the kininogen and the factors participating in the release process.

In experiments with acid-treated human plasma, HAMBERG (1962) and WEBSTER & PIERCE (1963) found that plasma kallikrein released mainly bradykinin whereas HABERMANN & BLENNEMANN (1964) obtained the same result with acid treated kininogen from bovine serum and casein activated hog-serum kallikrein Recently however ELLIOTT LEWIS & SMYTH (1963) and ELLIOTT & LEWIS (1965) were able to isolate methionyl-lysyl-bradykinin from the acid treated pseudoglobulin fraction of bovine serum after a spontaneous activation, and ARMSTRONG & MILES (1963 1964) reported on the release of hitherto unknown kinins from the euglobulin precipitate from human plasma not submitted to acid.

In experiments with human or bovine plasma substrates and different glandular kallikreins WERLE, TRAUTSCHOLD & LEYSATH (1961), WEBSTER

& PIERCE (1963) and HABERMANN & BLENNEMANN (1964) all concluded that the main kinin primarily released was probably identical with kallidin.

The reports on the effect of trypsin are not entirely conclusive about the identities of the released kinins. The experiments of ELLIOTT HORTON & LEWIS (1960 1961) suggested that mainly bradykinin was released by trypsin from the euglobulin precipitate from ox serum, but ELLIOTT (1963) pointed out that trypsin might be able to break down kallidin to bradykinin PIERCE & WEBSTER (1961) and WEBSTER & PIERCE (1963) obtained as bradykinin nearly 100 / of the kinin released by trypsin from human plasma. At the same time, however they stressed the ability of trypsin *to cause a slow conversion of kallidin to bradykinin and also showed that* aminopeptidases were present in the plasma. HABERMANN & BLENNEMANN (1964) found that trypsin released mainly bradykinin in their acid treated bovine kininogen but also some kallidin. In view of parallel experiments with trypsin and hog pancreas kallikrein the last mentioned authors discussed the possible presence of two separate kininogens. Previous work also reflects uncertainty about the homogeneity of plasma kininogen (WERLE, KEHL & KOBKE 1950 VAN ARMAN 1955 HABERMANN 1963 FASCIOLO 1963)

Our own work was carried out as experiments preliminary to the establishment of a method for the determination of kininogen in human plasma. Human citrated plasma, and in some experiments also guinea pig and rat plasma, were heated at pH 2 for 30 minutes at 100° or for 10 minutes at 37° or phenanthroline was added as a stabilizing agent, without acidification Trypsin and hog pancreas kallikrein were used as kinin-releasing enzymes. The kinins released were tested on the isolated rat uterus, and in some basic experiments their kinin nature was confirmed by parallel assays on the isolated rat duodenum.

No direct identification of the released kinins was attempted some indirect evidence, however was obtained through conversion experiments with aminopeptidase The interpretation of such experiments was based on the known molecular weights and relative biological activities of kallidin and bradykinin.

Technique

A. *Materials and methods*

1 *Plasma kininogen substrates* Human blood was collected by venipuncture int siliconized bottle containing 10 ml of 3 1 % sodium citrate dihydrate solution per 90 ml blood, immediately transferred t silico ized centrifuge bottles and centrifuged at 4 × 10³G for 30 minutes t 10° The plasma was divided int 3 portions.

Substrate I was prepared by adding 0 5 mg phenanthroline in 0.03 ml of water per ml of citrated plasma, with subsequent heating for 10 minutes at 37° The substrate was stored at −20° in 1 ml samples, corresponding to 0 88 ml plasma.

Substrate II was prepared by adding about 0.2 ml f 1 N hydrochloric acid per ml of citrated plasma to pH 2, with subsequent heating for 10 mi utes at 37° in waterbath. The substrate was stored at −20 in 1 ml samples, correspond ng to 0.75 ml plasma.

Substrate III was acidified in the same manner as substrate II, diluted with 4 ml of 0.9 % sodium chloride, heated for 30 minutes at 100 in a waterbath and stored t −20° in 5 ml samples, corresponding to 0.75 ml plasma.

2. *Kininogen determinations*. *Substrate I* was incubated with releasing enzymes without pH-adjustment, nd *substrates II and III* were brought in ad nce t pH 8 by adding bout 7 ml 0.1 N sodium hydroxide. Incubations with trypsin were usually carried out with 0.5 mg enzyme per ml citrated plasma. If plasma was found to contain less than 3 μg of bradykinin per ml, the assay was repeated with trypsin also (pre-treated as in the test solutions) dded to the standard solutions. The amounts f the different enzymes used are shown in the T bles.

The kinin assays were generally carried out on the isolated rat uterus as "bracketing assays" with a standard dose ratio f 3 2. Bradykinin was used as standard substance. I some experiments parallel assays on the isolated rat duoden m were used to confirm that the substances stim lating rat uterus released from plasma were in fact kinins (GADDUM & HORTON 1959 HORTON 1959).

3. *Chemicals*.

Aminopeptidase Leucine aminopeptidase, LAP 5982, Worthingto Biochemical Corp., Freehold, New Jersey U.S.A. *Bradykinin* (synthetic) in ampoules of 100 μg/ml, Sandoz, A.G., Basel, Switzerland. *Padutin* ® (hog pancreas kallikrein). In ampoules of 10 biol. units, Bayer A.G., Leverkusen, Germany *1 10-Phenanthroline* o-Phenanthroliniumchlorid, Merck, A.G Darmstadt, Germany *Trypsin*, twice cryst., washed free f salt TRSF Worthington Biochemical Corp. Freehold, New Jersey U.S.A.

B. *Comment on th materials and methods*

1 *Plasma kininogen sub trates*. Phenanthroline has been used as *in vitro* inhibitor of kininases by ERDÖS & SLOANE (1962) and ERDÖS, RENFREW SLOANE & WOHLER (1963). The last-named observed strong inhibition of both human plasma kininase and human erythrocyte kininase at inhibitor molar concentration of 3 10^{-4} Later DYRUD, RINVIK & BRISEID JENSEN (1965) found phenanthroline effective against the same kininases at the same or somewhat lower concentrations. I ur work the concentration chosen for substrate I was about 2 10^{-3}M

GADDUM & HORTON (1959) inactivated plasma kininase by adding hydrochloric acid to a pH somewhat below 2 nd then incubating for 10 minutes at 37 This procedure has since been widely used nd was adopted in our work for substrate II

VAN ARMAN (1955) nd later HAMBERG & ROCHA E SILVA (1957) prepared plasma substrates by adding an equal volume of 0.2 % acetic acid and heating to 80–90° for some minutes in a waterbath. The acidification and heating proced re dopted in our work for substrate III is even more vigorous.

Kininogen determinations. The conditions chosen for the kininogen determinations are discussed by RINVIK, DYRUD & BRISEID (1966).

Results

A. *Kinin release by trypsin*

Table 1 shows that the plasma substrate heated for 30 minutes at 100° and pH 2 (substrate III) released no kallidin on incubation with trypsin. No increase in activity was observed on subsequent incubation with aminopeptidase the results might suggest that only bradykinin was set free. If the temperature during the acid treatment was lowered to 37° and the time reduced to 10 minutes (substrate II), a mixture of bradykinin (or other kinins not convertible with aminopeptidase) and kallidin (or other kinins convertible to bradykinin with aminopeptidase) was set free.

Table 1

Comparison of the release by trypsin f kinins from huma plasma substrates stabilized by addition of phenanthroline, by heating t 37° or 100° at pH 2.

Plasma substrate I 0.5 mg phenanthroline/ml citrated plasma and 10 minutes at 37
Plasma substrate II Hydrochloric acid to pH 2 and 10 mi tes at 37°
Plasma substrate III Hydrochloric acid t pH 2 and 30 mi utes at 100°
Trypsin 0.5 mg/ml fresh plasma.
Aminopeptidase 1 5 µg/ml fresh plasma.
The incubations were carried ut at pH 7 4 and 6 for substrate I, at pH 8 and 37° for substrates II nd III. For all substrates the fractions were then heated at pH 1 boiling wate bath for 5 min tes a d assayed on the isolated rat uterus.

Plasma substrat	Plasma specimen	µg kinin per ml fresh plasma as bradykinin after incubation with trypsi 30 minutes	µg kinin per ml fresh plasma as bradykinin after incubation with trypsin 30 mi utes aminopeptidase 30 minutes
I	1	6.3 (2 mi utes)	–
II	1	5.0	6.3
	2	–	–
	3	–	
	4	–	
	5	4.0	4 6
	6	4.7	5.3
III	1	6.3	6.3
	2	5.0	–
	3	5.0	
	4	4.2	
	5	4.6	4 6
	6	5.3	5 4

Table 2

Significance f pH during the preparation f plasma substrate fo the relative amounts eleased by trypsi of the kallidin – and bradykinin fractions.

Plasma substrate Hydrochloric acid to pH 2 and 10 ml utes t 37 Then hydrochloric acid or sodium hydroxide to different pH values and 100° f 30 minutes. Control sample without pH adjustment nd 100 heating.
Trypsin l mg/ml plasma.
The incubations were carried out f 30 mi utes at pH 8 a d the fractions were then heated at 100 f 5 minutes and tested on the isolated rat uterus.

pH	1.5	2	3	4	5	7	8	Co trol sample
µg kinin pe ml plasma as bradykinin	6.3	6.3	6.3	5.6	5.0	5.0	5 0	5 0

On addition of aminopeptidase the uterus-activity of the released kinins increased to the level obtained in substrate III. The results might indicate that the kininogen of plasma during the acid treatment was altered so as to release bradykinin on trypsin incubation Some conversion already seemed to have taken place during the 37° incubation, and heating at 100° caused a complete transformation of the substrate. If a sample of substrate II was adjusted to pH 7 4 and then heated for 30 minutes at 100° the same kinin-value was obtained as with untreated substrate II indicating that the pH and not the temperature was primarily responsible for the kininogen conversion Experiments were accordingly carried out to investigate roughly at what pH value the heating of plasma at 100° would cause increased quantities of bradykinin to be released on trypsin incubation The results are shown in table 2. Though the kinin activity at pH 5 was still the same as that observed at pH 7 and 8 some kininogen transformation had taken place at pH 4 At pH 3 and at lower values the conversion seemed to have been complete.

Evidence of the kinin nature of the uterus-stimulating substances released from substrates II and III was obtained from parallel assays on rat duodenum (GADDUM & HORTON 1959)

Table 1 shows, for one specimen, that the same kinin activity was found in the phenanthroline-stabilized plasma (substrate I) as in substrate III and in substrate II on addition of aminopeptidase. The results suggest that no significant loss of kininogen took place during the acid-boiling process used for substrate III The results presented in table 1 might also indicate that trypsin directly released bradykinin in the phenanthroline plasma (substrate I) especially as model experiments carried out with

phenanthroline and leucine-aminopeptidase showed a complete inhibition of the enzyme at the inhibitor concentration used

However the experiments did not exclude the possibility that the aminopeptidases of *plasma* were active to some extent in the presence of phenanthroline, thereby converting to bradykinin the kallidin eventually released by trypsin

In the experiments with substrate I we used a low incubation temperature (26°) to restrict the effect of small amounts of the kininase still present. In spite of this low temperature, the kinin was released considerably more rapidly than in the partly denaturated acid-treated substrates. After 1 minute incubation about 80 / of the total activity was found, after 2 minutes 100 / after 5 minutes 80 / after 15 minutes 70 / and after 30 minutes 60 /

If the phenanthroline concentration was increased from 0 5 mg to 2.0 mg per ml plasma, the inactivation of kinin was significantly delayed At the same time, however the phenanthroline seemed to inhibit the release of kinin by trypsin, a lower maximum value being obtained. Also an increase in trypsin concentration resulted in lower recoveries, probably through an activation of kinin splitting enzymes.

B *Kinin release by hog pancreas kallikrein (padutin ®)*

It is well known that less kinin will be released by hog pancreas kallikrein than by trypsin both in human plasma (FASCIOLO & HALVORSEN 1964) and in bovine plasma (HABERMANN & BLENNEMANN 1964).

Accordingly the results in table 3 compared with those in table I show that padutin only partly exhausted the plasma kininogen of substrate II After conversion by aminopeptidase of the kallidin fractions released, the resulting bradykinin fractions amounted to about 70 / of the bradykinin fractions resulting from trypsin release and aminopeptidase transformation (plasma specimens 1 5 and 6) When the padutin and aminopeptidase incubated samples (table 3) were further treated with trypsin more bradykinin was set free, the final amounts corresponding roughly to those in the trypsin and aminopeptidase incubates (table I).

Table 3 further shows that the yield of kinin by incubation of substrate II with padutin gradually decreased with increasing incubation time. After 20 hours only about 20 of the initial activity was left, and subsequent further incubation with trypsin caused only a small additional release of kinin The loss of activity was not due to contaminants in the padutin preparation itself as a parallel experiment with plasma heated at 100° and pH 2 did not show a decrease in kinin. It is more probable that the heating at pH 2 for 10 minutes at 37 did not completely inactivate the

Table 3

Comparison of the stepwise release by padutin ® (hog pancreas kallikrein) and trypsin of kinins from human plasma substrates stabilized by heating at 37° or 100° at pH 2.
Plasma substrate II: Hydrochloric acid to pH 2 and 10 minutes at 37°
Plasma substrate III: Hydrochloric acid to pH 2 and 30 minutes at 100°
Trypsin 0.5 mg/ml fresh plasma.
Padutin 1 unit/ml fresh plasma. Addition of another unit for incubation periods over 120 minutes.
Aminopeptidase 1.5 µg/ml fresh plasma.
The incubations were carried out at pH 8, and the samples were then heated for 5 minutes at 100° and assayed on the isolated rat uterus or further incubated with another enzyme.

Plasma substrates	Enzymes	µg kinin per ml fresh plasma as bradykinin in plasma specimens: 1				2	3	4	5				6			
		after incubation time in hours														
		½	1	2	20	½	½	½	¼	½	1	2	¼	½	1	2
II	padutin	3.3	3.1	2.8	0.6	3.1	3.0	2.6	–	2.8	–	–	–	3.5	–	–
	+aminopeptidase	4.2	–	–	0.6	3.5	3.3	2.6	–	3.3	–	–	–	3.9	–	–
	+trypsin	6.0	–	–	1.6	4.9	4.3	3.6	–	4.7	–	–	–	5.8	–	–
	+ aminopeptidase	6.0	–	–	1.6	–	–	–	–	4.8	–	–	–	5.8	–	–
III	padutin	1.3	1.6	2.5	2.8	–	–	–	0.8	1.0	0.9	1.0	0.9	1.2	1.2	1.4
	+ aminopeptidase	–	–	–	2.8	–	–	–	–	–	–	1.0	–	–	–	1.2
	+trypsin	–	–	–	6.1	–	–	–	–	–	–	–	–	–	–	–

kininase present. Traces left of the enzyme might cause slow inactivation of the kinin gradually released. Experiments with plasma specimens from other subjects also indicate that the preparation procedure mentioned for substrate II will not always give a completely kininase-free substrate. In practice however only incubation times beyond 1 hour caused a significant decrease in the amounts of kinin determined.

When plasma had been heated for 30 minutes at 100° and pH 2 (substrate III) it was less suitable for kinin release by padutin. Though trypsin, in less than 30 minutes, caused a maximal release of bradykinin, padutin quickly (in about 15 minutes) set some kinin free, but the main part only slowly. Table 3 shows that after 20 hours incubation at 37° less than 50% of the theoretical amount was released in plasma specimen 1. Subsequent incubation with aminopeptidase suggested that only bradykinin had been set free, no increase in activity taking place. On the other hand, subsequent

Table 4

Release of bradykinin by trypsin and padutin ® (hog pancreas kallikrein) from guinea pig plasma substrates stabilized by heating at 37° or 100° at pH 2.
Plasma substrate II Hydrochloric acid to pH 2 and 10 minutes at 37°
Plasma substrate III Hydrochloric acid to pH 2 and 30 minutes at 100°
Trypsin 1 mg/ml fresh plasma.
Padutin 2 units/ml fresh plasma.
The incubations were carried out at pH 8, and the samples then heated for 5 minutes at 100° and assayed on the isolated rat uterus or rat duodenum or further incubated with another enzyme

Plasma substrat	Enzymes	–	¼	1	2	3	20
		µg kinin per ml fresh plasma as bradykinin after incubation time in hours					
II	trypsin	–	–	–	–	–	–
	+aminopeptidase	6.0	6.0 (rat duoden.)	–	–	–	–
	trypsin	–		–	–	–	–
	+aminopeptidase	5.9	5.9 (rat duoden.)	–	–	–	–
III	padutin	0.8	–	1.5	2.0	2.2	4.2 4.2 (rat duoden.)
	+ aminopeptidase	–	–	–	2.0	–	–

incubation with trypsin demonstrated that the kininogen was intact, the final amount of bradykinin being of the usual order

Discussion

Our experiments carried out with human plasma specimens stabilized by heating at acid pH were intended to provide a safer basis for a method for determining the kininogen The results obtained with substrates prepared by acid treatment at different temperatures and subjected to different enzymes suggested that heating at 100° at pH 3 or lower (1.5 was the lowest pH examined) would yield a kininogen that reacted as a homogeneous substance to trypsin The enzyme seemed to release one kinin only presumably bradykinin Such a substrate, which is rather similar to the substrate described by VAN ARMAN (1955) reacted less readily to padutin R (hog pancreas kallikrein) but obviously also set free bradykinin when incubated with that enzyme (table 3). In this connection we report our observation that heating at 100° at low pH also made the plasma kininogens of the guinea pig and the rat accessible to padutin. According to FASCIOLO & HALVORSEN (1964), BRISEID JENSEN, RINVIK & VENNERÖD (1965) and BRISEID JENSEN DYRUD & RINVIK (1965) plasma specimens from those species do not release kinins on incubation with

padutin Table 4 shows how padutin set kinin free from guinea pig plasma substrate prepared by heating for half an hour at 100 and pH 2 (substrate III) Just as was observed for human plasma (table 3), the release was slow and as the activity of the kinin fraction was not increased through incubation with aminopeptidase the product was judged to be bradykinin only Evidence of the kinin nature of the active substance was provided by parallel assays on the isolated rat duodenum As was noticed with human plasma, the yield of kinin released by trypsin was the same as for the substrate prepared at 37° (II) indicating that no loss of kininogen took place during the boiling with acid. Experiments with rat plasma specimens also showed that heating at pH 2 and 100° rendered a substrate accessible to the effect of padutin

Though the heating at 100° at pH 2 (substrate III) altered the kininogen so that it yielded one kinin only the results shown in tables 1 to 3 indicate the presence in substrate II (37° at pH 2) of two kininogens, one of which was a conversion product of the other and was produced by the acid treatment. Substrate III released only bradykinin, whereas substrate II released a mixture, possibly consisting of kallidin and bradykinin Both trypsin and padutin acted rapidly to release the kallidin fraction from the altered part of the kininogen molecule, however trypsin set free bradykinin readily but padutin only acted slowly to release bradykinin The fact that padutin from substrate II released a kinin fraction that, like kallidin was convertible to bradykinin by incubation with aminopeptidase supports the view commonly expressed in the literature that glandular kallikreins will release kallidin from plasma kininogen As mentioned earlier in this paper it is not, on the other hand, generally accepted that trypsin sets kallidin free to a considerable extent, as suggested by our results.

It should be pointed out that the kinin fraction rapidly released by padutin did not consist exclusively of kallidin Calculations based on the aminopeptidase transformation experiments summarized in table 3 on the known relation of the molecular weights of bradykinin and kallidin (about 0 9) and on the relative uterus activities (about 0 6) showed that a significant part of the kinin must have been released as bradykinin This gives some evidence of the presence of another kininogen besides the main one, more or less altered by the acid heating procedure at least there is an indication of the existence of one kininogen that does not react as if it were homogeneous

Summary

Human citrated plasma acidified to pH 2 and heated for 10 minutes at 37° on incubation with trypsin released a mixture of kallidin (or other

kinins convertible to bradykinin with aminopeptidase) and bradykinin (or other kinins not convertible with aminopeptidase) If the acidified plasma was heated for 30 minutes at 100 the kininogen was transformed so as to release one kinin only presumably bradykinin. The transformation did also take place at pH 3 and partially at pH 4 but in the pH range 5–8 the heating at 100 caused no demonstrable conversion of the kininogen. No significant loss of kininogen took place during the heating at 100 and pH 2.

Hog pancreas kallikrein (padutin ®) also released a kinin mixture, consisting probably of kallidin and bradykinin, from citrated plasma acidified to pH 2 and heated at 37° but the total amount of kinins was less than that released by trypsin One reason for the smaller yield was that the part of kininogen transformed by heating with acid was less accessible to the glandular kallikrein. In accordance with this the main bulk of bradykinin was released only slowly and incompletely through padutin from citrated plasma acidified to pH 2 and heated for 30 minutes at 100 This heating with acid also made the plasma kininogens of the guinea pig and the rat, normally not accessible to padutin slowly releasable by that enzyme

The kinin fractions were tested on the isolated rat uterus and their kinin nature confirmed by parallel assays on the isolated rat duodenum.

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Acta pharmacol et toxicol 1966 24 50–54

From the Pharmaceutical Biological Laboratory
Royal Danish School of Pharmacy Copenhagen

The Antidiuretic Effect of 5-Hydroxytryptamine in the Rat after Subcutaneous Injection of 'Physiological' Doses

By

Per Frandsen and Aage Theil Nielsen

(Received October 19 1965)

About ten years ago ERSPAMER and collaborators (ERSPAMER & OTTOLENGHI 1953 ERSPAMER 1954 ERSPAMER & CORREALE 1955) demonstrated an antidiuretic effect of 5-hydroxytryptamine (5HT), when injected *subcutaneously* in hydrated rats. The smallest active dose was about 4 µg/kg. Clearance measurements indicated that the effect was due to constriction of the afferent glomerular arterioles. ERSPAMER considered the response physiological' since about 5 µg/kg of endogenous 5HT is metabolized by the rat every half hour For some years he regarded 5HT as a hormonal substance participating in the regulation of the intrarenal circulation of the mammalian kidney This theory however has not been supported by experiments on other species, including man Except in rats, the antidiuretic effect of 5HT is variable and obtained only with high unphysiological doses (cf PAGE 1958 ERSPAMER 1961).

Although ERSPAMER's observations on rats have been confirmed by other authors (ARMAN & JENKINS 1956 DASGUPTA 1957) opinion is still divided as to their physiological significance. The objection has been advanced that the effect of subcutaneously injected 5HT is merely artefactual, an indirect effect of pain at the site of injection (CORCORAN *et al* 1954 PAGE 1954 THORN 1958) It is well known that 5HT at high dilution (10^{-8}) produces pain when applied to the exposed human dermis (ARMSTRONG *et al* 1953), and pain may elicit antidiuresis *via* the neurohypophysis or by reflex renal vasoconstriction (PAPPENHEIMER 1960 THEOBALD & VERNEY 1935 THEOBALD 1934) This conception fits well with the fact, also noted by ERSPAMER, that 5HT does not inhibit water excretion when injected intraperitoneally in similar doses.

Table 1

Rate of water excretio in hydrated rats after subcutaneous injection of 5HT Four rats/group V lume injected 1 ml/kg of body weight.

Time of excretion of 45 % of water load (minutes)

Control Solution Sodium chloride 0.9 %		Test Solution 5HT 10 µg/ml	
Group I	Group II	Group III	Group IV
77	93	120	101
72	67	91	86
95	98	112	125
81	98	103	111
82	100	121	103

Mean: 86.3 mi utes Mean 107.3 minutes
Delay 107.3 − 86.3 = 21.0 minutes
t-test of significance P < 0.001

In this paper a reinvestigation of the antidiuretic effect in the rat after subcutaneous and intraperitoneal injection is reported. To test the hypothesis that the antidiuretic effect of 5HT after subcutaneous injection is indirect and due to local sensory stimulation, experiments were per formed in which a local anaesthetic was added to the solution of 5HT

Methods

Female albino rats weighing between 200 and 230 g were used. Food was removed from the animals o the evening before an experiment was to be carried out. Next morning 16 animals were given 5 ml/100 g of tepid water by stomach tube and distributed at random into f ur cages for urine collection, 4 rats in each cage. Two groups of 4 rats were injected with 0.2 ml of control solution and two similar groups with 0.2 ml of test solutio (for details see tables 1–4). The solutions were made isotonic to blood by ddition of sodium chloride. The urine was collected, and the amounts were recorded at 15 mi utes intervals until excretion had nearly stopped. The point of maximum rat of excretion was determined graphically as the point at which 45 % of the water dministered had been excreted. Each experiment was repeated once week on the same stock of nimals. For testing the significance of the difference between the effects of control and test solutions the 'treatment x day' interaction mean square was used as the error variance.

Doses of 5HT re expressed as base.

Results

Effect of 5HT

The experiments recorded in table 1 show a significant (P < 0.001) antidiuresis when 10 µg/kg of 5HT were injected subcutaneously In

Table 2

Rate of water excretion in hydrated rats after intraperitoneal injection of 5HT Four rats/group. Volume injected 1 ml/kg of body weight.

Time of excretion f 45 % of water load (minutes)

Control Solution Sodium chloride 0.9 %		Test Solution 5HT 20 µg/ml	
Group I	Group II	Group III	Group IV
80	107	99	97
108	97	94	95
114	97	100	94

Mean 100.5 minutes Mean 96.5 minutes
Delay 96.5 − 100.5 = −4 minutes
t test of significance P > 0 4

contrast, 20 µg/kg were inactive (table 2) when injected intraperitoneally (P > 0 4)

Effect of procaine on response to 5HT

Rats were injected subcutaneously with 10 µg/kg of 5HT with 2 % of procaine hydrochloride added to the solution No antidiuretic effect (P > 0.9) was noted when compared with that on animals injected with a control solution of 2 % of procaine hydrochloride (table 3)

To investigate whether the suppressant effect of procaine on the response to 5HT was local or systemic, the experiments summarized in table 4 were carried out. They demonstrate that the usual response to 5HT was unaffected by the simultaneous injection of procaine, when the substances are injected separately at two symmetrical sites of the skin on the back (P < 0.001)

Discussion

The present results confirm ERSPAMER s observations on the antidiuretic effect of 5HT in microgram doses given subcutaneously to rats and the lack of effect when similar doses are injected intraperitoneally ERSPAMER explains the difference between the effects after subcutaneous and intraperitoneal injection by assuming that the antidiuretic effect of 5HT depends on the slow rate of absorption after subcutaneous injection Our experiments, however demonstrate that procaine suppresses the anti

Table 3

Rate of water excretion in hydrated rats afte subcutaneous injection f 5HT with procaine. Four rats/group. Volume injected 1 ml/kg of body weight.

Time of excretion f 45 % of water load (minutes)

Control Solution Procaine hydrochloride 2 % (w/v)		Test Solution Procaine hydrochloride 2 (w/v) + 5HT 10 µg/ml	
Group I	Grup II	Group III	Group IV
82	89	90	99
82	80	78	76
86	81	87	91
83	9	88	76
71	56	60	60

Mean 80 2 minutes Mean 80 5 minutes
Delay 80.5 − 80.2 = 0.3 minutes
t-test f significance $P > 0.9$

Table 4

Rate of water excretion in hydrated rats after 5HT and procaine, injected separately at two symmetrical sites f the kin of the back. Fou rats/group. Vol me injected 1 ml/kg of body weight.

Time of excretion of 45 % of water load (minutes)

Control Solutions Left site Sodi m chloride 0.9 % Right site Procaine hydrochloride 2 % (w/v)		Test Solutions Left site 5HT 10 µg/ml Right site Procaine hydrochloride 2 % (w/v)	
Group I	Group II	Group III	Group IV
99	98	106	109
81	90	125	115
86	97	87	98
80	73	106	102
95	112	99	119
88	105	122	97
94	78	96	116

Mean 91 1 minutes Mean 106.9 minutes
Delay 106.9 − 91 1 = 15.8 minutes
t-test of significance $P < 0.001$

Table 2

Rate of water excretion in hydrated rats afte intraperitoneal I jec tio of 5HT Four rats/group. Volume injected 1 ml/kg of body weight.

Time of excretion of 45 of water load (minutes)

Control Solution Sodium chloride 0.9		Test Solution 5HT 20 µg/ml	
Group I	Gr up II	Group III	Gro p IV
80	107	99	97
108	97	94	95
114	97	100	94

Mea 100.5 mi utes Mean 96 5 minutes
Delay 96.5 − 100 5 = −4 min tes
t-test of significance $P > 0.4$

contrast, 20 µg/kg were inactive (table 2) when injected intraperitoneally ($P > 0 4$)

Effect of procaine on response to 5HT

Rats were injected subcutaneously with 10 µg/kg of 5HT with 2 % of procaine hydrochloride added to the solution No antidiuretic effect ($P > 0 9$) was noted when compared with that on animals injected with a control solution of 2 / of procaine hydrochloride (table 3)

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From the Research Laboratories of AB Astra, Södertälje, Sweden

Inhibition of Liver Phenylalanine and Tryptophan Hydroxylating Enzyme Systems In Vitro and in Vivo

By

S. B. Ross and Ö. Haljasmaa

(Received September 2, 1965)

α-Methyl-DOPA and some dihydroxyphenylacetamide derivatives decrease the level of 5-hydroxytryptamine (5-HT) and 5-hydroxyindole acetic acid (5-HIAA) in the brains of rats and mice, indicating inhibition of the enzymatic hydroxylation of tryptophan to 5-hydroxytryptophan (5-HTP) (Roos & Werdinius 1963 Carlsson, Corrodi & Waldeck 1963 Carlsson & Corrodi 1964) The phenylalanine and tryptophan hydroxylating enzyme system in rat liver has been shown to be inhibited by these compounds *in vitro* (Burkardt Gey & Pletscher 1964 Ross & Haljasmaa 1964) and *in vivo* (Burkardt Gey & Pletscher 1964)

In the study reported here we investigated the *in vitro* and *in vivo* inhibition of the liver phenylalanine and tryptophan hydroxylases activated by different cofactors. Four of the inhibitors (esculetin, α-propyl-3 4-dihydroxyphenylacetamide (H 22/54), α-methyl DOPA and aminopterin) were studied in more detail

Methods

Phenylalanine and tryptophan hydroxylase. Crude liver extract was obtained by homogenizing rat liver with 3 volumes of ice-cold 0.067 M phosphate buffer pH 7 4 and centrifuging at 27 000 g for one hour at +2° (Burkardt Gey & Pletscher 1964). The preparations were stored at −17° and used within few days.

A partially purified enzyme was prepared from rat liver by the method of Kaufman (1957), in which the purification was stopped after the first ammonium sulphate precipitation. The precipitate was dissolved in 0.033 M Tris buffer pH 6.8, and dialysed against this buffer

An enzyme preparation of phenylalanine hydroxylase from rat liver was also made by the method of Mitoma (1956). Purified factor 1 and crude factor 2 were used in the hydroxylation assay

The phenylalanine and tryptophan hydroxylase activities were determined by the method of UDENFRIEND & COOPER (1952a) a d FRIEDLAND, WADZINSKI & WAISMAN (1961a), respectively In the phenylalanine hydroxylase assay 0.5 ml f the enzyme solutio was incubated with 2 µmol L-phenylalanine, 5 µmol nicotinamide 2 µmol of the activator used and any inhibito to be tested. The final volume was 1 35 ml. The incubation was performed by shaking for one hour t 22 The tryptophan hydroxylase assay was performed with 1 ml of the enzyme solution, 40 µmol L-tryptopha 5 µmol nicoti amide, 2 µmol of the activator used nd any inhibitor to be tested. The final olume was 2.5 ml and the incubation mixture was shaken at 37 for ne hour Every enzyme assay was performed in duplicate. Heat denat red enzyme was used in the control for non-enzymatic hydroxylation. The reaction was termin ted with 1 ml of 20 / trichloroacetic acid. The amount of tyrosine formed was determined in the centrif ged extract by the method of UDENFRIEND & COOPER (1952b) and 5-hydroxylndoles formed by the method f UDENFRIEND WEISSBACH & CLARK (1955).

The activators of the hydroxylases used were nicotinamide-adenine dinucleotide (NAD), reduced nicotinamide-adenine dinucleotide (NADH), reduced nicotinamide-adenine dinucleotide phosphate (NADPH) and 2 amino-4-hydroxy-6,7-dimethyl-5,6,7 8-tetrahydropteridine (DMPH)

The kinetic studies were perf rmed with the crude extract and with the partly purified rat-liver enzyme, with $DMPH_4$ as activato The incubation time for the phenylalanine hydroxylase assay was 5 minutes with 5 minutes preincubati before ddition of the activator The substrate concentrati ns used were 1.5×10^{-4} 3×10^{-4} 6×10^{-4} and 1.6×10^{-3} M The Michaelis constants and the inhibitor constants were evaluated by the methods of LINEWEAVER & BURK (1934) and DIXON (1953).

5-hydroxytryptamine in mouse brain was extracted and determined by the method of BOGDANSKI *et al.* (1956)

Results

Phenylalanine hydroxylase

The time curves for the phenylalanine hydroxylase activity of the crude liver extract activated by the different cofactors are given in fig. 1 In all

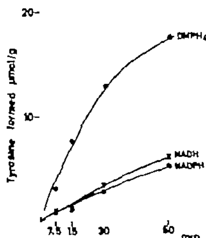


Fig. 1 T me curves for phenylala ine hydroxylase activities of crude rat-liver extract with various cofactors.
The incubation conditions were the same as those described in the legend to table 1

Table 1

Phenylalanine hydroxylase activity of rat-liver extract activated by different cofactors.

The incubation mixture was 0 5 ml f the extract, 2 µmol of L-phenylalanine, 5 µmol of nicotinamide, 2 µmol f the cofactor used. The final volume was 1.35 ml. The incubatio was conducted t 22 for one hour

Extract No.	Tyrosine formed µmol/g/h			
	NAD	NADH	NADPH	$DMPH_4$
1	3.9	4.6	2.4	9 1
2	1.4	1 1	2.0	2.1
3	–	5.2	3 4	5.9
4	6.4	8.7	7.3	8.3
5	4.9	5 0	6.7	9 6
Mean	4.2	4 9	4.4	7 0

experiments $DMPH_4$ had the highest activating effect at high concentrations the three nicotinamide adenine dinucleotides studied had about equal activities (table 1) The concentration response for NADH and NADPH was, however different (fig. 2) Although these cofactors activated the hydroxylation in catalytic amounts, higher concentrations of NADPH than of NADH were needed to obtain maximal effect. This finding indicates a higher level of NAD reducing enzymes in the extract Fig. 2A demonstrates that at the incubation temperature of 37° the simultanous activating effect of NADH and NADPH was additive at low

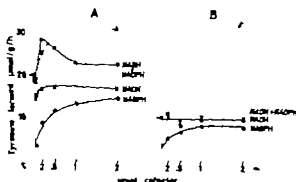


Fig. 2. Phenylalanine hydroxylase activities with different mounts of NADH and NADPH.

Incubation was performed at A 37° and B 22° The dotted line represents the estimated sum of the effect of NADH nd NADPH. Other incubation conditions were the same as those described in the legend to table 1

Table 2

Effect of preincubation of crude rat-liver extract with oxygen at 37° on the phenylalanine hydroxylase activity with different cofactors.

The preincubation was conducted for 30 minutes with oxygen at 37°. The other experimental conditions were the same as those described in the legend to table 1.

Experiment *No.*	Tyrosine formed µmol/g/h							
	Non-preincubated extract				Preincubated extract			
	NAD	NADH	NADPH	$DMPH_4$	NAD	NADH	NADPH	$DMPH_4$
1	6.4	8.7	7.3	8.2	0.3	1.3	3.4	2.8
2	4.9	5.0	6.7	9.4	0.1	1.3	3.2	7.4

concentrations, but was lower than the estimated sum at higher concentrations. At room temperature the hydroxylase activity with the simultaneously acting cofactors did not exceed that with NADH alone at any concentration studied (fig. 2B).

UDENFRIEND & COOPER (1952a) found that preincubation of crude rat liver extract with oxygen at 37° for half an hour almost completely eliminated the NAD activating effect on phenylalanine hydroxylation, whereas the NADP-activated hydroxylation decreased to about half of its initial activity. These results were confirmed by us, and we also found that the $DMPH_4$ activated hydroxylation decreased somewhat under this condition (table 2). UDENFRIEND & COOPER (1952a) found, too, that storing the extract in a refrigerator for some days considerably decreased the NADP-activating effect on hydroxylation, whereas the effect of NAD was only slightly decreased. As is shown in table 3, we were able to confirm these results and found as well that the activating effect of NADPH and $DMPH_4$ remained after this treatment of the liver extract.

Inhibition in vitro

Esculetin was the most potent inhibitor of the three compounds tested on the phenylalanine hydroxylation when the enzyme reaction was activated by the nucleotides (fig. 3 and table 4), confirming previous results (ROSS & HALJASMAA 1964). It was, however, a considerably weaker inhibitor of the $DMPH_4$-activated hydroxylation reaction compared with the nucleotide activated reaction. When the extract preincubated in oxygen

Table 3

Effect of refrigerator storage of the rat-liver extract on its phenylalanine hydroxylase activity

The frozen rat liver extract was thawed and stored in refrigerator (+3°) for 36 hours. The hydroxylase activity was determined as described in the legend to table 1 and compared with that of freshly thawed extract of the same preparation.

Activator	Tyrosine formed µmol/g/h	
	Refrigerator extract	Fresh extract
NAD	5.6	7 1
NADH	6 9	6.9
NADP	0.6	2.5
NADPH	4.1	5.7
$DMPH_4$	7.3	7 6

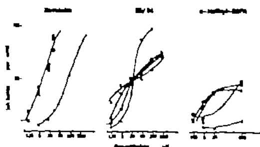


Fig. 3 Inhibition of the phenylalanine hydroxylase activity of crude rat-liver extract *in vitro*.
The incubation conditions were the same as those described i the legend to table 1 The hydroxylation reaction was activated by 1.5×10^{-3}M f NAD O, NADH Δ, NADPH ● or $DMPH_4$ ×

Table 2

Effect of preincubation of crude rat-liver extract with oxygen at 37 on the phenylalanine hydroxylase activity with diff rent cofactors

Th preincubation was conducted for 30 minutes with oxygen at 37° The other experimental conditions were the same as those described in the legend to table 1

Experiment No	Tyrosine formed µmol/g/h: Non-preincubated extract				Preincubated extract			
	NAD	NADH	NADPH	$DMPH_4$	NAD	NADH	NADPH	$DMPH_4$
1	6.4	8 7	7.3	8.2	0 3	1.3	3 4	2.8
2	4 9	5 0	6.7	9 4	0.1	1.3	3.2	7 4

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Table [illegible]

Effect of refrigerator storage of the [illegible] extract on its phenylalanine hydroxylase activity

The frozen rat liver extract was [illegible] and kept in a refrigerator (+3) for 24 hours. The [illegible] activity was determined as described in the legend to table [illegible] and compared with that of [illegible] extract of the same preparation.

Activator	Tyrosine formed [illegible]	
	Refrigerator extract	Fresh extract
NAD	3.6	7.1
NADH	6.9	6.9
NADP	0.6	2.5
NADPH	4.1	5.7
$DMPH_4$	7.3	7.6

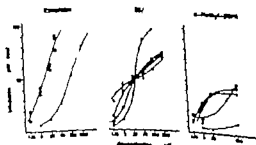


Fig. 3 Inhibition of the phenylalanine hydroxylase activity of crude rat liver extract *in vitro*.

The incubation conditions were the same as those described in the legend to table 1. The hydroxylation reaction was activated by 1.5×10^{-3}M of NAD O NADH Δ, NADPH [illegible] or DMPH ×

Table 2

Effect of preincubation of crude rat-liver extract with oxygen at 37° on the phenylalanine hydroxylase activity with different cofactors.

The preincubation was conducted for 30 minutes with oxygen at 37°. The other experimental conditions were the same as those described in the legend to table 1

Experiment No.	Tyrosine formed µmol/g/h — Non-preincubated extract				Preincubated extract			
	NAD	NADH	NADPH	$DMPH_4$	NAD	NADH	NADPH	$DMPH_4$
1	6.4	8.7	7.3	8.2	0.3	1.3	3.4	2.8
2	4.9	5.0	6.7	9.4	0.1	1.3	3.2	7.4

concentrations, but was lower than the estimated sum at higher concentrations. At room temperature the hydroxylase activity with the simultaneously acting cofactors did not exceed that with NADH alone at any concentration studied (fig. 2B).

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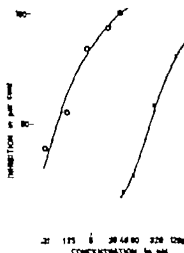


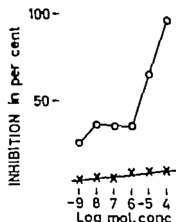
Fig. 4 Inhibiting effect of esculetin on the phenylalanine hydroxylase activity with crude liver extract (×) and with the partly purified enzyme (KAUFMAN 1957) (○)
Activator DMPH₄ (1.5 × 10⁻³M). Incubation conditions as described in the legend to table 1

Of the three compounds tested on the nucleotide-activated phenylalanine hydroxylation α-methyl-DOPA was the weakest inhibitor and had scarcely any effect on the $DMPH_4$-activated reaction at the concentrations used. Higher amounts of the compound than that causing about 50% inhibition could not be investigated, since it interfered with the tyrosine determination.

Aminopterin was shown by KAUFMAN & LEVENBERG (1959) to inhibit the pteridine reductase from sheep liver that is involved in the phenylalanine hydroxylating system. We found that at 10^{-4}M it had no effect on the $DMPH_4$-activated reaction, but considerably inhibited the NAD activated hydroxylation with crude rat liver extract (fig. 5). The concentration course of the inhibitory effect indicates that aminopterin at low concentrations (10^{-8}M) had a different mode of action on the hydroxylase system from that at higher concentrations.

Results of kinetic studies with different concentrations of the substrate, the activator and the inhibitor indicate that H 22/54 inhibited the $DMPH_4$-activated hydroxylation noncompetitively with the substrate (fig. 6) and the activator (fig. 7)[1]. Esculetin inhibited the hydroxylation competitively with the substrate (fig. 8). The inhibition constant (K_i) for H 22/54 was about $1-2 \times 10^{-5}$ M with either the crude extract or the partly purified enzyme. K_i for esculetin was 1.5×10^{-6}M with the preincubated crude

[1]) Although H 22/54 in most experiments reacted as a noncompetitive inhibitor we obtained in some experiments results that can be interpreted as an uncompetitive (coupling) inhibition



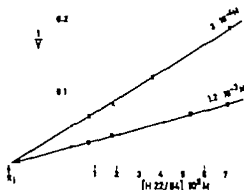
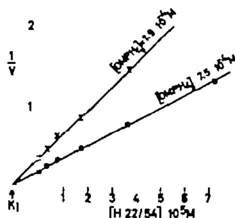


Fig. 5 Inhibiting effect f aminopteri on the phenylalanine hydroxylase activity of crude rat-liver extract.

The incubation conditions were as described i the legend to table 1 Activators 1.5 × 10⁻³M NAD O and DMPH4 × respectively

Fig. 6. I verted velocities of the phenylalanine hydroxylase activities (μmol/g/h tyrosine formed) plotted against the different concentrations of H 22/54 at two substrate concentrations.

The phenylalanine concentrations were 3 × 10⁻⁴M or 1.2 × 10⁻³M. The incubation as performed for 5 minutes at 37 Crude liver extract was used. Activator DMPH4 (1.5 × 10⁻³M).



F g 7 Inverted elocities of the phenylalanine hydro ylase activities (μmol/h tyrosine f rmed) plotted gainst the different amounts of H 22/54 at two concentrations of DMPH4.

The phenylalanine concentration wa 1.5 × 10⁻³M The incubation was conducted for 5 minutes at 37 Partially purified enzyme (Kaufman) was used.

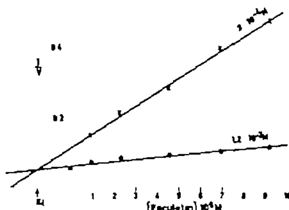


Fig. 8. Inverted velocities of the phenylalanine hydroxylase activities (µmol/g/h tyrosine formed) plotted against the different amounts of esculetin at two phenylalanine concentrations.
The conditions used were the same as those described in the legend to fig. 6.

extract and the partly purified enzyme. The Michaelis constant for this enzyme reaction was about 7×10^{-4}M with both the crude and purified enzymes.

Inhibition in vivo

Table 6 demonstrates the inhibition of the phenylalanine hydroxylase activity of mouse liver extract after injection of the compounds studied

Table 6

Inhibition of mouse-liver phenylalanine hydroxylase activity *in vivo*.

The enzyme activity was assayed *in vitro* with liver extract prepared as described in the legend to table 1. The compounds tested were injected intraperitoneally 30 minutes before the mice were killed. Each figure is the mean for three groups of three mice each.

Compound	Dose mg/kg	Per cent inhibition NAD	DMPH$_4$
Esculetin	200	68	72
	100	0	14
H 22/54	200	46	8
	100	0	–
α Methyl-DOPA	800	36	10
	200	24	
Aminopterin	100	79	6

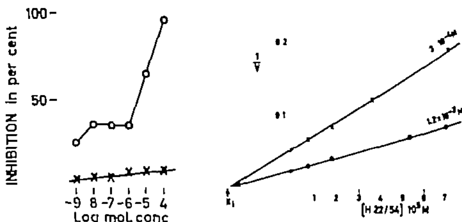


Fig. 5 Inhibiting effect f aminopterin on the phenylalanine hydroxylase activity of crude rat-liver extract.
The incubation co ditions were as described in the legend to table 1
Activators 1.5×10^{-3}M NAD O and $DMPH_4$ × respectively

Fig. 6. Inverted velocities of the phenylalanine hydroxylase ctivities (μmol/g/h tyrosine formed) plotted against the different concentrations of H 22/54 at two substrate concentrations.
The phenylalanine concentrations were $3 \times 10^{-4}M$ or $1.2 \times 10^{-3}M$. The incubation was performed for 5 minutes t 37 Crude liver extract was used. Activator $DMPH_4$ (1.5×10^{-3}M).

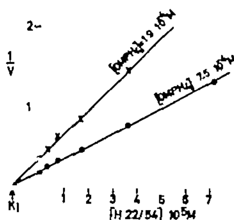


Fig 7 Inverted velocities of the phenylalanine hydro ylase activities (μmol/h tyrosine formed) plotted against the different amounts of H 22/54 at two concentrations of $DMPH_4$.
The phenylalanine concentratio was 1.5×10^{-3}M The incubation was conducted for 5 minutes at 37° Partially purified enzyme (Kaufma) was used.

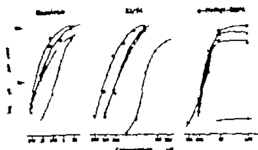


Fig. 9 Inhibition of the tryptophan hydroxylase activity of crude rat liver extract *in vitro*.

The incubatio conditions were the same as those described in the legend t table 7 The hydroxylatio was activated by 8 × 10^{-} M of NAD ○, NADH △ NADPH ● or DMPH ×

Table 8

Inhibitor concentrations givi g 50% inhibition f the tryptophan hydroxylase activity with different cofactors.

The concentrations giving 50% inhibition of th tryptopha hydroxylase activity were obtained from the values shown in figure 5

Compound	50 per cent inhibitory molar concentration			
	NAD	NADH	NADPH	$DMPH_4$
Esculetin	$3 \times 10^{-}$	$3 \times 10^{-}$	3×10^{-7}	2×10^{-4}
H 22/54	$3 \times 10^{-}$	1×10^{-4}	1×10^{-4}	3×10^{-4}
α-Methyl-DOPA	1×10^{-4}	1×10^{-4}	1×10^{-4}	$>4 \times 10^{-4}$

hydroxylation (fig. 9 and table 8) Esculetin and α methyl-DOPA inhibited the hydroxylation activated by the different nucleotides to about an equal extent, but H 22/54 seemed to have a somewhat greater effect on the NAD-activated hydroxylation than on that activated by the reduced nucleotides. Though α-methyl-DOPA had scarcely any effect on the $DMPH_4$ activated reaction, it was as active as the other two compounds on the nucleotide-activated hydroxylation of tryptophan Aminopterin had no effect on the $DMPH_4$-activated hydroxylation of tryptophan and inhibited the NAD-activated reaction at concentrations about equal to those affecting the phenylalanine hydroxylase (fig. 10) The concentration response was also similar to that obtained with phenylalanine as substrate. The compounds studied were almost equally active on the hydroxylation activated by $DMPH_4$ with the preincubated liver extract as with the un incubated extract.

Since tryptophan had a low affinity for the enzyme, the inhibitor con stants could not be determined

Table 9

Inhibition of mouse-liver tryptophan hydroxylase activity *in vivo*.

The enzyme activity of mouse-liver extract 30 *minutes* after injection of the compounds studied was measured as described in the legend to table 7. The figures are means of three groups of three mice each. The effect of the compounds on the 5-HT content of brain was kindly reported by Dr H. Corrodi, AB Hässle, Göteborg, Sweden except for the compounds marked with an asterisk.

– = not tested 0 = no effect on 5-HT content + = decrease of 5-HT content.

Part I

Compound	HO OH (ring)			Dose mg/kg i.p.	Per cent inhibition of tryptophan hydroxylase activated by NAD	Per cent inhibition of tryptophan hydroxylase activated by $DMPH_4$	Effect on 5-HT level in mouse brain
Esculetin	–O–COCH = CH–		H	200	100	27	
				50	56	–	0)
				25	42	–	
α-Methyl-DOPA	H	$CH_2C(CH_3)(NH_2)COOH$	H	800	–	19	
				100	78	–	+
				50	21	–	
Aminopterin			–	100	85	21	–
Caffeic acid	H	CH = CH–COOH	H	200	30	9	0)
H 17/47	H	$CH = CH–CONH_2$	H	200	44	34	0)
H 13/48	H	$CH_2CH_2CONH_2$	H	200	–	31	–

Part II

Compound	HO, OH-benzene ring substituents			Dose mg/kg i.p.	Per cent inhibition of tryptophan hydroxylase activated by		Effect on 5-HT level in mouse brain
					NAD	$DMPH_4$	
H 13/49	H	CH_2CONH_2	H	200	–	37	–
H 22/07	H	$CH(OC_2H_5)CONH$	H	200	–	37	+
H 22/21	H	$CH(CH_3)CONH$	H	200	–	17	+
H 22/41	H	$CH(C\ H_5)CONH$	H	200	–	25	+
H 22/44	H	$C(CH_3)_2CONH_2$	H	200	–	27	+
H 22/54	H	$CH(C_3H_7$-n$)CONH_2$	H	400		97	++
				200	80	63	+
				50	46	–	
H 22/58	H	$CH(C_4H_9)CONH_2$	H	200	–	33	+
H 22/59	H	$CH(C_3H_7$-iso$)CONH_2$	H	200	–	43	+
H 22/93	H	$CH(C_6H_{13})CONH_2)$	H	200	–	0	
H 17/77	H	H	CH_2CONH	200	54	34	(+)
				100	39	–	
H 33/07	H	H	$CH(OC_2H\ )CONH_2$	200	68	27	+
				100	40	–	
H 29/55	H	H	$CH(C_3H_7$-n$)CONH_2$	200	–	38	+
H 46/16	H	H	$CH(C_3H_7$-iso$)CONH_2$	200	–	48	+

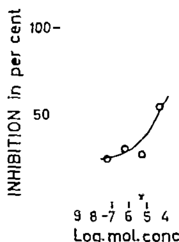


Fig. 10. Inhibiting effect of aminopterin on the tryptophan hydroxylase activity of crude rat-liver extract.
The incubation conditions were the same as those described in the legend to table 7
Activators 8×10^{-4}M NAD ○ o DMPH$_4$ ×

Inhibition in vivo

The *in vivo* effect of numerous compounds on the DMPH$_4$-activated tryptophan hydroxylase activity in mouse liver extract was studied (table 9) For some compounds the effect on the NAD-activated hydroxylation also was determined. The mice were killed half an hour after intraperitoneal injection of the compounds tested At this time H 22/54 had its maximal inhibiting effect the other compounds were not tested in this way The effect on the 5-hydroxytryptamine level in mouse brain after treatment with the compounds studied is also given in the table for comparison of the *in vivo* liver-enzyme inhibiting effects (most of the values were kindly communicated by Dr H Corrodi see also Carlsson, Corrodi & Waldeck 1963 Carlsson & Corrodi 1964) The table demonstrates that those compounds inhibiting the DMPH$_4$-activated liver tryptophan hydroxylase activity at the same dose level also decreased the 5-HT content of brain. An exception, however is α-methyl-DOPA, which had almost no effect on the enzyme activity Some compounds inhibiting the NAD activated hydroxylation *in vivo* had no effect on the 5-hydroxytryptamine content of brain, *i.e* esculetin and aminopterin (Renson *et al* 1962) The most active inhibitors of the DMPH$_4$-activated tryptophan hydroxylation *in vivo* were H 22/54 H 22/59 and H 46/16. These compounds were also among those that had the highest effect on the 5-hydroxy tryptamine content of mouse brain (Carlsson, Corrodi & Waldeck 1963 Carlsson & Waldeck 1964)

Discussion

Several investigators have pointed out the similarities between the liver phenylalanine and tryptophan hydroxylating enzyme systems (FREEDLAND, WADZINSKI & WAISMAN 1961 RENSON *et al* 1961) However some differences between these two systems also seem to exist. FRIEDLAND (1963) found that Fe^{++} activated and EDTA suppressed the enzymatic hydroxylation of tryptophan, but these agents had no effect on the phenylalanine hydroxylation. GAL, CHATTERJEE & MARSHALL (1964) found that sodium borohydride ($NaBH_4$) inhibited the phenylalanine hydroxylation, but did not affect the tryptophan hydroxylation *in vitro* Our findings that α-methyl-DOPA and H 22/54 inhibited the nucleotide-activated phenylalanine hydroxylation differently from that of the tryptophan hydroxylation (fig. 3 and 9), whereas the inhibition of the $DMPH_4$-activated reactions followed the same type of responses, also indicate some differences between the two enzyme systems.

The compounds studied inhibited the nucleotide-activated phenylalanine hydroxylase with different concentration responses, as shown in fig. 3 The concentration curve of the inhibition with H 22/54 seems to be composed of two different curves, one similar to that obtained with α methyl-DOPA and the other to that with esculetin.

The partial inhibition of the nucleotide-activated phenylalanine and tryptophan hydroxylases by low concentrations of aminopterin (fig. 5) may be related to its inhibitory effect at this concentration level on dihydrofolic reductase, which according to KAUFMAN (1963) may be involved in the phenylalanine hydroxylase system. The increase in the inhibition of the hydroxylases at considerably higher concentrations of aminopterin may on the other hand, be related to its inhibiting effect on pteridine reductase (KAUFMAN & LEVENBERG 1959)

The lack of inhibitory effect of α-methyl-DOPA on the $DMPH_4$-activated hydroxylation of phenylalanine and tryptophan indicates that this compound inhibits the nucleotide-activated enzyme systems at a stage preceeding the last pteridine-dependent hydroxylation reaction. At least two enzymes are involved at this stage of the system, *i.e* a pteridine reductase (KAUFMAN & LEVENBERG 1959) and NAD or NADPH reducing enzymes. Either α-methyl-DOPA may inhibit one of these enzymes, or the reaction with the naturally occurring pteridine, boipterin (KAUFMAN 1963), may be more sensitive to α methyl-DOPA than that with the $DMPH_4$ used in our investigation.

Although esculetin and H 22/54 inhibit the $DMPH_4$-activated hydroxylases, these compounds also may interfere with the nucleotide-activated systems at a different site, as discussed above for α methyl-DOPA. The

considerably higher activity of these compounds on the nucleotide activated tryptophan hydroxylation than on the $DMPH_4$-activated reaction may afford support for this assumption

The increase in sensitivity of the $DMPH_4$-activated phenylalanine hydroxylase to esculetin with purification or preincubation of the crude extract may not be explained by loss of an esculetin metabolizing factor since the sensitivities of the nucleotide activated reactions were almost unchanged

RENSON *et al* (1961) and RENSON, WEISSBACH & UDENFRIEND (1962) suggested that the liver tryptophan hydroxylating systems has no function in the formation of 5-HT in the organs containing it. In spite of this, the *in vivo* effects of the dihydroxyphenylacetamide derivatives studied on the liver tryptophan hydroxylase-activated by $DMPH_4$ and on the 5-HT content of brain seem to show some relationship In contrast to esculetin, H 22/54 inhibits the phenylalanine hydroxylase noncompetitively with the substrate and with $DMPH_4$ It is possible that the other acetamide derivatives also are noncompetitive inhibitors of this enzyme and that compounds of this type had the same mode of action on other hydroxylases involving a pteridine derivative as cofactor

The phenylalanine hydroxylase activity with NADP after storage of the extract in a refrigerator found by UDENFRIEND & COOPER (1952a) may be due to a decrease in the enzymes reducing NADP since the enzyme activity with NADPH was only slightly affected by this storage of the extract.

Summary

The inhibition of the phenylalanine and tryptophan hydroxylating enzyme systems from rat liver extract by esculetin a propyl-3 4-dihydroxyphenylacetamide (H 22/54) a Methyl DOPA and aminopterin was investigated with nicotinamide adenosine dinucleotides (NAD NADH NADPH) and 2-amino-4-hydroxy-6 7-dimethyl 5 6 7 8-tetrahydropteridine ($DMPH_4$) as enzyme activators.

Although a methyl-DOPA inhibited the nucleotide-activated hydroxylation of tryptophan completely and that of phenylalanine in part, it had hardly any effect on the $DMPH_4$-activated hydroxylations.

Esculetin and H 22/54 inhibited the hydroxylations of both amino acids with all the activators used, but generally the $DMPH_4$-activated reactions were less sensitive to the inhibitors than the nucleotide-activated hydroxylations. Whereas H 22/54 was a noncompetitive esculetin was a competitive inhibitor of the $DMPH_4$-activated phenylalanine hydroxylation.

Aminopterin inhibited the NAD-activated hydroxylation of the amino acids at about equal concentrations but had no effect on the $DMPH_4$-activated reaction

The activity of mouse-liver tryptophan hydroxylations was investigated after treatment of mice with some tryptophan hydroxylase inhibitors, with NAD and $DMPH_4$ as activators. Of the inhibitors on the $DMPH_4$-activated hydroxylation H 22/54 was the most potent. Esculetin, α-methyl-DOPA and aminopterin, being good inhibitors of the NAD-activated hydroxylation, had considerably less effect on the $DMPH_4$-activated system in this *in vivo – in vitro* system.

Acknowledgements

We thank Dr H Corrodi AB Hässle, Göteborg, Sweden for generous supplies of α methyl-DOPA, H 22/54 and 2-amino-4-hydroxy-6,7 dimethylpteridine. For the reduction of the latter compound to $DMPH_4$ we thank Dr R Sandberg, AB Astra, Södertälje, Sweden.

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From the Research Laboratories of AB Astra, Södertälje, Sweden

In Vitro Inhibition of Noradrenaline-³H Uptake by Reserpine and Tetrabenazine in Mouse Cerebral Cortex Tissues

By

S. B. Ross and A. L. Renyi

(Received Oct ber 1 1965)

DENGLER, SPIEGEL & TITUS (1961 a & b) and DENGLER *et al* (1962) *investigating the in vitro uptake of tritiated noradrenaline by different* tissues from the cat found that reserpine was a potent *in vitro* and *in vivo* inhibitor of the uptake.

In this paper we report studies of the mechanism of the reserpine inhibiting effect on the uptake of tritiated noradrenaline by use of mono-amineoxidase inhibitors, tetrabenazine and other compounds interferring with the metabolism of noradrenaline.

Methods

Uptak of tritiated noradrenaline

The *in vitro* uptake of tritiated noradrenaline by the cortex tissue of mouse brain was determined by the method of DENGLER *et al.* (1962) with some minor modifications (ROSS & RENYI 1964). The incubation mixture was 100 mg cortex slices, 2 ml Krebs-Henseleits solution, 0.1 mμmol noradrenaline-7-³H (3.6 C/mmol, New England Nuclear Corp.) and any compou d t be tested. The atmosphere was 93.5 % O_2 and 6.5 % CO_2, the temperature 37 nd the incubation time one hour I the extraction th procedure was that the slices were rapidly wiped on filter paper and weighed and were then homogenized with 2 ml of bsolute ethanol in small centrif ge tubes. A 0 1 ml portio of the incubation medium was added to 2 ml of ethanol. All extracts were centrifuged half an hour later and the radio-activity in 0.5 ml of the alcohol-extract was counted in liquid scintillation system (Packard Tri Carb Autoanalyzator). The scintillation liquid used was 10 ml of a toluene-ethanol (9 1) solution of 0.4 % 3,4-diphenyloxazole and 0.01 % β-bis-[2-(phenyloxazolyl)]-benzene. The ratio of radioactivity concentration in the slices to that in the medium (R) was taken as measure of the uptake of noradrenaline. The percentage blocking effect of the compounds tested was calculated by the formula

$$\frac{(R_c - R_i)100}{R_c - 1}$$

where R_c is the ratio of the control and R the ratio i the presence of the inhibitor or the treated animal. Every assay was performed at least in triplicate.

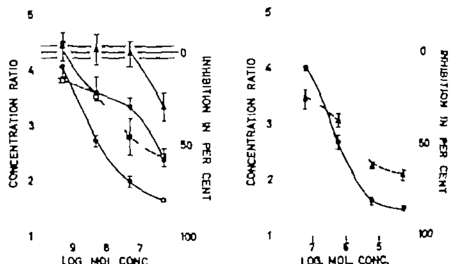


Fig. 1 Inhibition f noradrenaline-³H uptak by reserpine *i vitro* with or without pretreatment by MAO-inhibitors N pretreatment ○ Phenelzine hydrochloride, 10 mg/kg i.p. △ Tranylcypromine sulfate, 10 mg/kg i.p ● Pargyline hydrochloride 60 mg/kg i.p. □. The mice were treated three hours bef re the experiment. The slices were incubated with *reserpine* nd 0 1 *mμmol/ml noradrenaline-³H hydrochloride in* Krebs-Henseleit solution for one hour The poi ts plotted re means for t least four slices.

Fig. 2 Inhibition f noradrenaline-³H uptak by *tetrabenazine i vitro.*
U treated mice ○ Phenelzine hydrochloride 10 mg/kg i.p. △ Experimental conditions were the same as described in the legend to fig. 1

Motor activity i mice

The exploratory m tor activity in mice was tested i a locomotion cage (Ärnström, Brunnberg & Ross, unpublished). The mice were tested for a ten minute period nd the same mouse was used for o ly one activity test. The inhibition of motor activity as calculated as percentage of the activity of saline-treated animals.

Results

Reserpine and tetrabenazine actions

The concentration responses of the *in vitro* inhibitory effect of reserpine and tetrabenazine on noradrenaline uptake was determined (fig. 1 and 2). Reserpine was about 100 times more potent than tetrabenazine. The inhibitions of noradrenaline uptake to 50 / were obtained at 5 10^{-9}M and 5 10^{-7}M respectively

Given *in vivo* each compound also had a marked inhibitory effect on the uptake of the amine (fig 3) The mice in this experiment were killed 18 hours after the reserpine injection and half an hour after the tetrabenazine treatment, respectively at these times the compounds were

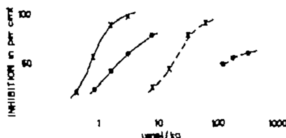


Fig. 3 Dose response f the inhibition of oradrenaline-³H uptak nd of pontaneous motor activity by reserpine and tetrabenazine in mice *in vivo*.

Moto activity × Noradrenalin ptak ○ Reserpine ——— Tetrabenazine ------- The experiments were perf rmed 18 hours after the reserpine injection and half an hou after the tetrabenazine injection The slices were incubated with 0.1 mμmol/ml noradrenaline-³H for one hour The points plotted are means for at least four animals.

strongly active. The dose-response curves for the inhibitory action of the compounds on motor activity in mice were determined in comparison with the effect on noradrenaline uptake (fig. 3) It is seen that reserpine was somewhat more potent in inhibiting the motor activity than the uptake reaction (ED50 0 45 and 1.3 mg/kg i.p respectively). For tetra benazine this difference was considerably larger (ED50 6 and 50 mg/kg i.p., respectively)

The time-curve for the reserpine action (fig. 4) shows that the inhibition persisted at a high level for one day and began to decrease during the second day The reserpine-induced sedation in mice kept at a room tempera

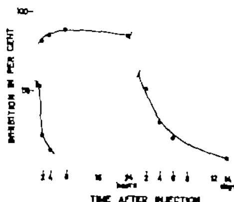


Fig. 4 Duration of the inhibitio f noradrenaline-³H uptake by reserpine and tetrabenazine in mice *i vivo*

Reserpine, 5 mg/kg i p. ● Tetrabenazine, 60 mg/kg i.p ○ The poi ts plotted are means for determinations from t least f ur animals.

ture of 26 showed a similar time course At a lower room temperature the sedation persisted much longer

The duration of the effect of tetrabenazine on noradrenaline uptake was much shorter than that of reserpine (fig. 4), consistently with the duration of other pharmacological and biochemical activities of this compound (PLETSCHER, BESENDORF & BÄCHTOLD 1958)

Interaction between tetrabenazine and reserpine

Tetrabenazine was shown by QUINN SHORE & BRODIE (1959) to counteract in rabbits the long acting pharmacological and 5-hydroxy tryptamine-depleting effects of reserpine when given shortly before reserpine, indicating a competition for the same site on the reserpine "receptors" The depletion of noradrenaline from the brain was, however not antagonized.

We found that pretreatment of the animals with tetrabenazine counter acted in part to about 50 / the *In vivo* reserpine-induced inhibition of noradrenaline uptake (table 1) Tetrabenazine, 80 mg/kg i p., was injected half an hour before reserpine, 5 mg/kg i.p., and the mice were killed 18 hours later *In vitro* however tetrabenazine had hardly any antagonizing effect on the irreversible reserpine inhibition of the amine

Table 1

I vivo counteraction of reserpine induced inhibition f noradrenaline uptake by tetrabenazine. Four animals were used for each determination.

Pretreatment	Dose, mg/kg p	Hours before experiment	Concentration ratio (± s.e.m.)	Inhibition of uptake (%)
—		–	3 9 ± 0.2	
Reserpine	5 mg/kg i.p.	18	2.1 ± 0.1	62
Tetrabenazine	80 mg/kg i.p.	18.5	3 7 ± 0.2	7
Tetrabenazine + Reserpine	80 mg/kg i.p. / 5 mg/kg i.p	18.5 / 18	3.0 ± 0.1	31

Table 2

Interaction between tetrabenazine and reserpine on the uptake of tritiated noradrenaline *in vitro*.

The slices were preincubated with the compounds studied for 15 minutes and washed in fresh incubation medium for 5 minutes, and the uptake of tritiated noradrenaline was continued in fresh medium for 45 minutes.

The figures are means for four slices.

Pretreatment	Concentration µg/ml	Concentration ratio (± s.e.m.)	Inhibition of uptake (%)
–	–	4.1 ± 0.5	–
Tetrabenazine	2	3.3 ± 0.1	26
Reserpine	0.03	1.3 ± 0.2	74
Tetrabenazine + Reserpine	2 0.03	2.2 ± 0.1	61

uptake (table 2). In this experiment the slices were pretreated simultaneously with tetrabenazine and reserpine and washed before the amine uptake. This also shows that the inhibitory effect of tetrabenazine is reversible.

MAO-inhibitors

Pretreatment of animals with MAO-inhibitors counteract the pharmacological and biochemical effects of reserpine (BRODIE, PLETSCHER & SHORE 1956, GIARMAN & SCHANBERG 1959, HILLARP & MALMFORS 1964). We found that pre-incubation of cortex slices with pheniprazine – a potent MAO-inhibitor – counteracted in part the reserpine induced inhibition of noradrenaline uptake (fig. 5). The same results were also obtained when the tissues were treated with pheniprazine after reserpine incubation or simultaneously with reserpine. In these experiments the noradrenaline uptake was allowed to proceed in a fresh incubation medium, since pheniprazine itself has an inhibitory effect on the uptake reaction when present in the medium. As shown in the figure, it had no effect under the conditions used.

Tetrabenazine-induced inhibition of the noradrenaline uptake was not

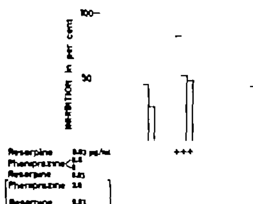


Fig. 5 Counteraction of the reserpine induced inhibition of noradrenaline-³H uptake by pheniprazine *in vitro*

The slices were washed after the preincubation which lasted for 15 minutes. The uptake of tritiated noradrenaline was performed in fresh medium for 45 minutes. Four slices were used in each experiment.

Table 3

Inhibitory effect of tetrabenazine on noradrenaline uptak by pheniprazine-treated tissue slices.

The slices were preincubated for 15 minutes with pheniprazine, washed nd incubated with tetrabenazine and tritiated noradrenaline fo 45 minutes.

The figures are means f r four slices.

Treatment	Concentration µg/ml	Concentration ratio (± s.e m)	Inhibition of uptake (%)
—	–	4.1 ± 0.15	
Pheniprazine	0.5	4.4 ± 0.12	0
Tetrabenazine	2	1.9 ± 0.03	71
Pheniprazine + Tetrabenazine	0.5 2	.2 ± 0.05	61

significantly antagonized by preincubation of the slices with pheniprazine (table 3)

When the MAO-inhibitor was given *in vivo* three hours before killing the mice, marked counteraction of the reserpine response *in vitro* was obtained (fig. 1) Pheniprazine had a considerably larger effect than tranylcypromine and pargyline in the doses used.

Table 4

Counteracting effect of three MAO-inhibitors on reserpine-induced inhibition of noradrenaline uptake. The MAO-inhibitors were injected six hours before reserpine, 5 mg/kg i.p. The mice were killed 18 hours later. The inhibition figures are based on experiments with four animals for each dose.

MAO-inhibitor	Dose mg/kg i.p.	Inhibition of uptake (%)
Pheniprazine	0	77
	0.4	62
	2	12
	5	11
	10	0
Tranylcypromine	0	77
	0.4	78
		63
	10	4
	20	29
Pargyline	0	79
	30	50
	60	0

The *in vitro* inhibitory effect of tetrabenazine was on the other hand only slightly counteracted by pretreatment of the animals with pheniprazine (fig. 2).

The potencies of the *in vivo* counteracting effect of three MAO-inhibitors on the uptake inhibitory action of reserpine was studied in a series of experiments. The MAO-inhibitors were injected six hours before reserpine 5 mg/kg i.p., and the animals were killed 18 hours later. Pheniprazine was slightly more active than tranylcypromine under these conditions (table 4) whereas pargyline was only about one fortieth as potent as pheniprazine.

The result of pheniprazine pretreatment of mice on the effect of reserpine on noradrenaline uptake when the animals were killed half an hour after the reserpine injection was investigated in another experiment (table 5). Under this condition pheniprazine also antagonized a great part of the acute inhibitory effect of reserpine on the amine uptake, but the counteraction was not so complete as it was 18 hours after the reserpine injection.

The tetrabenazine-induced inhibition of noradrenaline uptake was in

Table 5

Effect of pheniprazine on reserpine- or tetrabenazine-i duced inhibition of noradrenaline ptake by cortex slices. Pheniprazine was given fo hours before reserpine or tetrabenazine. The mice were killed half an hour later
Th figures re means for four animals.

Pretreatment	Dose mg/kg i p.	Concentration rati (± s.e.m.)	Inhibition of uptake ( )
–	–	3.8 ± 0.13	-
Reserpine	5	1 9 ± 0.07	68
Pheniprazine + Reserpine	10 5	3 0 ± 0.09	29
–	–	3 6 ± 0 15	
Tetrabenazine	60	2.3 ± 0.11	50
Pheniprazine + Reserpine	10 60	2.5 ± 0.10	42

contrast to the effect of *reserpine* being hardly at all counteracted by of the animals with pheniprazine under the same conditions (table 5) The sedating effect of tetrabenazine was, however antagonized by pheniprazine at the same dose

The duration of the MAO-inhibitors counteracting effect on amine uptake is shown in fig. 6. Pheniprazine, 10 mg/kg i p., had a marked effect when injected as long as five days before the reserpine administration Tranylcypromine had a somewhat shorter duration of action and did not antagonize reserpine as completely as pheniprazine. The figure also shows that when the MAO-inhibitors were given simultaneously with or after the reserpine administration they only counteracted 50/ or less of the reserpine response

PLETSCHER & BESENDORF (1959) and HORITA & McGRATH (1960) have demonstrated that pretreatment of amines with a short acting MAO-inhibitor e g harmaline, counteracts the long-lasting effect of a MAO-inhibitor with irreversible action. Consistently with this we found that harmaline, 2 mg/kg i.p given half an hour before pheniprazine also

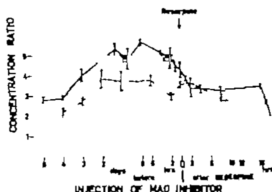


Fig. 6. Duration of the counteracting effect of phenelprazine a d t anylcypromine on reserpine-induced inhibition of noradrenaline-³H uptake mice.

Reserpine, 5 mg/kg i p., was given t 0 hour and the mice were killed 18 hours later Phenelprazine hydrochloride, 10 mg/kg i.p. ——— nd tranylcypromine sulphate, 10 mg/kg i.p. —— were injected into f ur nimals at the time indicated, before o after the reserpine injection. The noradrenaline-³H uptak was continued for one ho r. The concentration ratios (± s.e.m.) of the control animals and of the reserpine treated animals are given by the horizontal lines.

counteracted the effect of pheniprazine on the reserpine-induced inhibi tion of noradrenaline uptake (table 6)

Table 6

I he cou teraction of the pheniprazine antagonizing effect on reser pine-induced inhibition f noradrenaline uptake by cortex slices by harmaline.

Harmaline, 2 mg/kg i.p., was i jected half n hour before pheniprazine, 10 mg/kg i p. Reserpin was given six hours later nd the mice were killed 18 hours after th reserpine injection

The figures are means f f ur animals.

Treatment	Concentration ratio (± s.e.m.)	Inhibition of uptake (%)
—	4.8 ± 0.3	
Reserpine	1.5 ± 0.2	87
Pheniprazine + Reserpine	4 3 ± 0 2	13
Harmaline + Pheniprazine + Reserpine	.2 ± 0.2	69

Table 7

Co nteracti g effect of chlorpromazine on pheniprazine antagonizing actio on the reserpine-induced inh bition of tritiated noradrenaline uptake.

The figures are means fo four nimals.

Pretreatment	Dose mg/kg i.p.	Hours before experiment	Concentrati- on ratio (± s.e.m.)	Inhibition of noradrenaline uptake (%)
–	–	–	7.3 ± 0.35	
Reserpine	5	18	2.5 ± 0.22	77
Chlorpromazine	20	24	6.9 ± 0.29	6
Chlorpromazine + Reserpine	20 5	24 18	.9 ± 0.09	68
Chlorpromazine + Phen prazine + Reserpine	20 10 5	24 23 18	4.3 ± 0.26	48

High doses of chlorpromazine counteract the amine increase induced by MAO-inhibitors, but have no effect on MAO-inhibition (EHRINGER, HORNEKIEWICZ & LECHNER 1960) We found that chlorpromazine (20 mg/kg i p) injected half an hour before pheniprazine partly antagonized the effect of pheniprazine on reserpine (table 7)

3-Iodo-L tyrosine is a potent tyrosine hydroxylase inhibitor *in vitro* (GOLDSTEIN & WEISS 1965) Since inhibition of this enzyme may cause a decrease in catecholamine content of the brain in pheniprazine-treated mice also we investigated the effect of 3-iodotyrosine on the pheniprazine counteraction of reserpine induced inhibition of amine uptake. Iodotyrosine was given shortly after the pheniprazine injection and also two hours after pheniprazine, whereas reserpine was injected three thours after pheniprazine administration In this experiment the brain stem of the mouse was used instead of cerebral cortex, since the former contains considerably more of the catecholamines than the latter Table 8 demon strates that the concentration ratio with this part of the brain is higher than with cortex. 3-Iodotyrosine completely antagonized the effect of pheniprazine and also antagonized the pheniprazine-induced reversal of the sedating effect of reserpine

Table 8

Counteracting effect of 3-iodo-L-tyrosine on pheniprazine antagonizing action on the reserpine-induced inhibition of noradrenaline uptake by slices from the brain stem of mouse.

3-Iodo-L-tyrosine, 250 mg/kg i.p., were given in two injections, one shortly after and the other two hours after pheniprazine, 10 mg/kg i.p. Reserpine, 5 mg/kg i.p., was injected three hours after pheniprazine.

The figures are means for four animals.

Pretreatment	Concentration ratio (± s.e.m.)	Inhibition of uptake (%)
Pheniprazine + Reserpine	9 0 ± 0 4	
Pheniprazine + 3-Iodo-L tyrosine	9.5 ± 0.3	0
3-Iodo-L-tyrosine	[illegible] ± 0.5	10
Reserpine	3.8 ± 0.5	65
Pheniprazine + 3-Iodo-L-tyrosine + Reserpine	2.8 ± 0.4	78

α-Methyl DOPA and α-methyl-noradrenaline

α-Methyl-DOPA antagonizes some of the pharmacological effects of reserpine (NEČINA 1962) and in part the noradrenaline-depleting effect of reserpine (PFEIFER & GALAMBOS 1965). In the dose studied (400 mg/kg i.p.) it also counteracted in part the reserpine induced inhibition of noradrenaline uptake when given half an hour or six hours before reserpine (table 9). α-Methyl DOPA itself had only a slight inhibitory effect on the amine uptake under the conditions used. Since α-methyl-DOPA *in vivo* is metabolized to α-methyl-dopamine and α-methyl-noradrenaline (CARLSSON & LINDQVIST 1962), the effect of preincubating slices with α-methyl-noradrenaline before reserpine was studied. The slices were washed before incubation with tritiated noradrenaline since α-methyl-noradrenaline is itself a potent inhibitor of the uptake (ROSS & RENYI 1964). Table 10 shows that α-methyl-noradrenaline antagonized the reserpine effect *in vitro*.

Discussion

It may be pointed out that all the radioactivity soluble in ethanol in the slices was recorded, *i.e.* both the noradrenaline and its metabolites present in the tissues. DENGLER *et al.* (1962) found that about 70 % of the

Table 9

In vivo counteraction of reserpine-induced inhibition of noradrenaline uptake in cortex slices by α-methyl-DOPA.

α Methyl-DOPA, 400 mg/kg i.p., was given half an hour (exp. 1) and six hours (exp. 2) before reserpine, 5 mg/kg i.p. The mice were killed 18 hours later

Experiment No.	Treatment	Number of animals	Concentration ratio (± s.e.m.)	Inhibition of uptake (%)
1	–	4	4.4 ± 0.2	–
	Reserpine	4	1.5 ± 0.1	83
	α Methyl DOPA	5	3.2 ± 0.[illegible]	27
	α Methyl DOPA + Reserpine	8	2.3 ± 0.1	57
2	–	4	6.6 ± 0.41	
	Reserpine	4	2.2 ± 0.06	79
	α Methyl DOPA	4	6.0 ± 0.38	11
	α Methyl-DOPA + Reserpine	4	4.5 ± 0.21	38

Table 10

Effect of α-methylnoradrenaline on the reserpine-induced inhibition of noradrenaline uptake.

The slices were preincubated with α-methylnoradrenaline for 15 minutes with reserpine in fresh medium for 15 minutes, and the noradrenaline uptake was continued in fresh medium for 45 minutes. The control slices were incubated in the same manner but without the addition of any test compound.

The concentration ratio figures are means for four slices.

Treatment	Concentration µg/ml	Concentration ratio (± s.e.m.)	Inhibition of uptake (%)
–		3.5 ± 0.05	
Reserpine	0.03	2.5 ± 0.06	45
α-Methylnoradrenaline	1.0	4.3 ± 0.28	
α Methylnoradrenaline + Reserpine	1.0 0.03	3.9 ± 0.16	12

radioactivity taken up by cat cerebral cortex slices was in the intact amine. The metabolites, on the other hand, accumulated in the medium. These authors concluded that any noradrenaline taken up is rapidly bound and thereby protected from catabolism, since inhibition of MAO has no appreciable effect on the uptake. This rapid uptake and binding of nor adrenaline by slices may be compared with the uptake and binding of noradrenaline released from nerve endings, which seems to be the prin cipal biological means of inactivating the amine (WHITBY AXELROD & WEIL MALHERBE 1961)

The results obtained in our investigation indicate that the action of reserpine on the uptake of tritiated noradrenaline by cerebral cortex tissues *in vitro* is similar to or identical with the action of reserpine causing release and depletion of catecholamines from the tissues. This assumption is supported by four results. 1) The *in vivo* doses of reserpine inhibiting the uptake reaction and those causing depletion of catecholamines are of the same order 2) MAO-inhibitors counteract both types of reserpine action 3) Tetrabenazine, which depletes catecholamines in brain, also inhibits the uptake of noradrenaline. 4) Tetrabenazine counteracts the long-acting irreversible *in vivo* effect of reserpine in both types of action

If this assumption is correct reserpine does not act on the transport uptake of the tritiated noradrenaline through cell membranes but on the intracellular binding and storage of the amine LINDMAR & MUSCHOLL (1964), investigating the uptake of noradrenaline in perfused heart *in vitro* found that in this organ reserpine blocked at the noradrenaline storage sites. Results obtained by histochemical determination of nor adrenaline also support this mode of action of reserpine *in vivo* (HILLARP & MALMFORS 1964).

The antagonizing effect of MAO-inhibitors on the reserpine-induced long-lasting inhibition of noradrenaline uptake seems to be partly caused by the MAO inhibition itself and also in part by the increased amounts of catecholamines resulting from the MAO-inhibition When the MAO-inhibitor is administrated after reserpine or when slices are pretreated with the MAO-inhibitor *in vitro* the enzyme inhibition alone may explain the partly reversing effect on the reserpine-induced inhibition of the amine uptake under the experimental conditions *in vitro* the oxidative deamina tion of the tritiated noradrenaline is then inhibited

To obtain the complete antagonizing effect on the reserpineinhibited uptake of the amine, the MAO-inhibitor had to be injected some time before the reserpine administration. CARLSSON, HILLARP & WALDECK (1963), investigating the effect of reserpine on catecholamine uptake by adrenal medulla granules *in vitro* found that the inhibiting effect was competitive with the amine. They suggested that the increased level of

catecholamines after MAO-inhibition may be essential to the antagonizing mechanism of the MAO-inhibitors on the reserpine-depleting action of the amines. Our findings on the uptake reactions support this assumption.

1) The counteracting effect of the tyrosine hydroxylase inhibitor 3-iodo-L tyrosine on the action of pheniprazine on the reserpine induced inhibition of amine uptake and on reserpine sedation may be explained by a decrease in the catecholamine level of the brain in spite of the MAO-inhibition.

2) α Methyl DOPA, metabolized in brain to α methyl-dopamine and α methyl-noradrenaline (CARLSSON & LINDQVIST 1962), which are not attacked by MAO counteracted partly the reserpine action This may be explained by an increase of these amines in the brain Preincubation of slices with α methyl-noradrenaline before reserpine also antagonized the inhibitory effect of the latter

3) Chlorpromazine counteracting the increase on amines caused by MAO-inhibitors but not the enzyme inhibition (EHRINGER, HORNEKIEWICZ & LECHNER 1960) partly antagonized the effect of pheniprazine on the reserpine induced inhibition of noradrenaline uptake

Although tetrabenazine antagonized the irreversible inhibitory effect of reserpine on the noradrenaline uptake, it differred from reserpine in some respects. Pheniprazine only slightly counteracted the action of tetrabenazine on the amine uptake, but completely reversed the sedating effect of tetrabenazine in mice. It may be that the sedating effect of tetrabenazine is due to interference with 5-hydroxytryptamine storage as suggested by QUINN, SHORE & BRODIE (1959) This assumption may also explain the large difference in the doses of the compound giving inhibition of motor activity and amine uptake in mice This difference could, however also be caused by a leakage of the reversible bound tetrabenazine into the incubation medium under the *in vitro* conditions used for the amine uptake reaction.

Summary

The inhibitory effect of reserpine and tetrabenazine *in vivo* and *in vitro* on the uptake of tritiated noradrenaline by cerebral cortex slices from the mouse *in vitro* has been investigated The interaction of other compounds on the effect of reserpine and tetrabenazine has also been studied.

The reserpine induced inhibition of noradrenaline uptake had a long duration, whereas the effect of tetrabenazine lasted only for a few hours.

Tetrabenazine counteracted in part the long-acting effect of reserpine *in vivo* but had no effect *in vitro*

MAO-inhibitors completely antagonized the effect of reserpine when

given *in vivo* before reserpine, but had only a partly antagonizing effect when given after the reserpine. *In vitro* also the MAO-inhibitor phenipra zine only partly counteracted the effect of reserpine. Pheniprazine had only a slight effect on the tetrabenazine induced inhibition of amine uptake

The short-acting MAO-inhibitor harmaline injected before pheniprazine antagonized its counteraction of the reserpine effect. Chlor promazine at a high dose had a similar effect. The tyrosine hydroxylase inhibitor 3-iodo-L tyrosine, also antagonized the effect of pheniprazine when given after the latter

α-Methyl DOPA partly antagonized the effect of reserpine when injected before reserpine. Preincubation of slices with α-methyl-noradrena line before reserpine partly antagonized the reserpine action *in vitro*

The results obtained indicate that reserpine interferes with the accumulation of noradrenaline by brain slices at the intracellular storage site. The irreversible (long acting) inhibitory effect of reserpine on the amine uptake is antagonized by an increased content of catecholamines in the brain, which is induced by for example, MAO-inhibitors and α-methyl-DOPA.

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Department of Pharmacology (Professor E. Bárány),
University of Uppsala, Sweden.

Effect of Synthetic Oestrogens and Analogues on the Uptake of Oestradiol by the Immature Mouse Uterus and Vagina

By

Lars Terenius

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The relation between chemical structure and oestrogenic activity has been the subject of extensive investigations. Around 1930 the important biphenolic oestrogens, diethylstilboestrol, hexoestrol and dienoestrol were synthetized by E. C. DODDS and coworkers. These compounds were structurally not related to the natural oestrogens but equalled them in biological activity Further work on this subject revealed other potent compounds, such as the triphenylethylene derivatives and the doisynolic acids (cf GRUNDY 1956) In an earlier communication (TERENIUS 1965) we examined the uptake of oestradiol by organs of immature mice under various conditions. Uterus and vagina accumulated oestradiol-³H, and this uptake could be saturated by carrier oestradiol Inhibition of the uptake by uterus and vagina had a distinct structure specificity in experiments with some steroid hormones, but the uptake by liver and skeletal muscle was unaffected We decided to investigate whether or not certain synthetic oestrogens and analogues also inhibited oestradiol uptake.

Material and Methods

The 17 β-oestradiol-6,7-³H was obtained from New England Nuclear Corporation. Its specific activity was 152 μc/μg. It was purified and made up in isotonic saline as described earlier (TERENIUS 1965). The hexoestrol and butoestrol isomers and analogues were prepared by the author The assignment of *erythro* and *threo* configurations to the desoxy

hexoestrol isomers is only tentative¹). Commercial preparations of vallestril ® and TACE ® were used.

Albino mice of the N M R.I strain, 16 to 17 days old, were used in all experiments. Their body weights ranged from 9 to 11 g. The synthetic oestrogens for the competitive experiments were injected subcutaneously in 0.1 ml olive oil. Three hours later (as a rule) 0.01 µg oestradiol-³H was injected subcutaneously in 0.1 ml isotonic saline at a separate site. One hour later the uterus and vagina were dissected free and weighed wet. Amounts of 20 mg tissue was cut out from liver and muscle (rectus femoralis). The contents of radioactivity of the organs and tissue samples were measured individually by the liquid scintillation technique, and the counts recorded were transformed to disintegrations/time and unit wet weight as described earlier (TERENIUS 1965). The chemical nature of the radioactivity in the organs was not studied.

The oestrogenic activities of the tested compounds were measured by the increase in uterus dry weight in mice of the same age. The test compounds were injected in 0.1 ml olive oil once daily for 3 days, and the animals were killed on the 4th day. Their uteri were dissected free, dried for 24 h at 100° and then weighed.

Trivial names and abbreviations

Oestradiol	17 β-oestradiol(oestra 1,3,5(10)-triene-3,17 β-diol) when not otherwise stated
Oestradiol-³H	17 β-oestradiol-6,7-³H
Hexoestrol	3,4-bis(p-hydroxyphenyl)-hexane
Desoxyhexoestrol	3-(p-hydroxyphenyl)-4-phenyl hexane
Butoestrol	2,3-bis(p-hydroxyphenyl)-butane
Vallestril ®	3-(6-methoxy 2-naphtyl)-2,2 dimethyl pentanoic acid
TACE ®	2-chloro-1,1,2 tris(p-methoxyphenyl)-ethylene

The content of radioactivity is expressed as disintegrations per minute/mg wet tissue (DPM/mg). In the figures mean values have been used.

U = uterus V = vagina L = liver M = skeletal muscle

Results

The meso form of hexoestrol is a potent oestrogen. It was about 100 times more uterotrophic than the *racemic* isomer when tested on our strain of mice (fig. 2). Various amounts of *meso* and *racemic* hexoestrol were injected in 0.1 ml olive oil 3 h before 0.01 µg oestradiol-³H. The *meso* form was a much more effective inhibitor of oestradiol-³H uptake by

¹) A comment on the stereochemistry of these compounds. Hexoestrol and butoestrol have two asymmetric carbons, which are alike, and linked to the same atoms or groups. There is one optically inactive *meso form*, and there are two optically active enantiomers, which in equal parts give a *racemic* form. On the other hand, desoxyhexoestrol has two different asymmetric carbons, which give 4 possible stereoisomers. They are all optically active and form two racemates, which are called *erythro* and *threo* forms. The *erythro* form of desoxyhexoestrol can be considered derived from *meso*-hexoestrol by removal of one phenolic oxygen and the *threo* form correspondingly from *rac*-hexoestrol. The *erythro* and *threo* configuration has been tentatively assigned on the basis of the compounds physicochemical properties. Work is in progress to assign the configuration unequivocally.

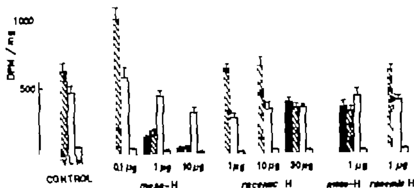


Fig. 1 Effect of the hexoestrol isomers on oestradiol-³H uptake i various organs.

A The hexoestrol isomers were dissolved i 0 1 ml oil and injected s.c. 3 h before the s.c. injection of 0.01 µg oestradiol-³H in 0.1 ml saline. The animals were killed 1 h fter the injection f the radioactive material.

B. The hexoestrol isomers were injected s.c. in 0.1 ml 10 % propylene glycol in saline *simultaneously with 0.01 µg oestradiol-³H in saline. Animals were killed 1 h later* There were 6–8 animals/group. Mean values are given vertical bars show one s.e.m. 1000 Dpm equals 2 8 × 10^{-3}% of the i jected dose.

uterus and vagina than the *racemic* isomer The amount of radioactivity in liver and skeletal muscle was not much affected (fig. 1A) In one experi ment 1 µg of the hexoestrol isomer was injected in saline simultaneously with 0 01 µg oestradiol-³H in saline at a separate site. Only the *meso* isomer inhibited oestradiol uptake by uterus and vagina (fig. 1B). Thus, there is a parallelism between uptake inhibition and uterotrophic activity

The same experiment was conducted with some closely related com pounds, whose dose response curves for uterotrophic activity are given in fig. 2. The desoxyhexoestrol isomers in ten µg doses stimulated oestra diol-³H uptake by uterus and vagina (fig. 3) This effect was most pronounced in the uterus, which reached almost twice the control level 3 h after injection. The uptake by uterus and vagina was significantly higher also 6 h after injection, but approached the control level 12 h after injec tion (table 1) At the 100 µg dose level and 3 h after injection the *erythro* isomer depressed oestradiol incorporation by uterus and vagina ($P < 0.01$) but the *threo* isomer which also is the less uterotrophic one, was without effect.

We investigated whether or not the stimulation of oestradiol-³H uptake in the uterus by the desoxyhexoestrol isomers also could potentiate its

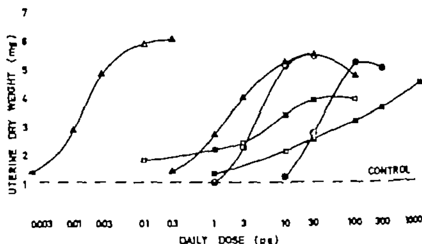


Fig. 2. Uterotrophic activity of the hexoestrol isomers and analogues. Dry terine weights after 3 injections on successive days. There were 5 nimals/group

—△—△— *meso*-hexoestrol
—▲—▲— *rac*-hexoestrol
—□—□— *meso*-b toestrol
—■—■— *rac*-butoestrol
—○—○— *erythro*-desoxyhexoestrol
—●—●— *threo*-desoxyhexoestrol

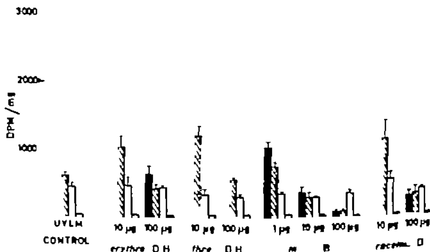


Fig. 3. Effect f the desoxyhexoestrol and butoestrol isomers oestradiol-3H uptake The test compound ere jected c oil 1 h before the injection of 0.01 μg oestradiol-3H (s.c.) in saline The nimals were killed 1 h la er There were 5–6 nimals/group Mea alues given ertical bars show one m.

DH deso yhexoestrol B butoestrol

Table 1

Effect of *erythro*-desoxyhexoestrol (*e*-DH) on uptake of oestradiol-^3H by various organs. At different times before 0.01 µg oestradiol-^3H in 0.1 ml isotonic saline 10 µg *e*-DH were injected in 0.1 ml olive oil. Animals were killed 1 h after injection of the radioactive material. The control animals received oil 3 h before the oestradiol-^3H injection. There were 6–7 animals/group. Mean values ± s.e.m. are given.

Interval between *e*-DH and oestradiol	Uterus		Vagina		Liver	Muscle
	wet weight (mg)	Dpm/mg wet weight	wet weight (mg)	Dpm/mg wet weight	Dpm/mg wet weight	Dpm/mg wet weight
Control (no *e*-DH)	8.6	1154 ± 52	7.4	675 ± 50	501 ± 47	50 ± 2.1
3 h	8.8	177 ± 157	8.1	1036 ± 163	494 ± 119	47 ± 4.9
6 h	8.3	2036 ± 142	8.1	1230 ± 158	5[illegible]1 ± 28	58 ± 12.3
1[illegible] h	12.9	1370 ± 92	8.0	1041 ± 67	591 ± 91	44 ± 5.4

biological action. An amount of 10 µg *threo*-desoxyhexoestrol, which has no oestrogenic effect of its own, was injected 3 h before 0.003 µg oestradiol. These injections were performed on 3 successive days, and the animals were killed on the 4th day (table 2). The pre-injection of *threo*-desoxyhexoestrol was found to increase the uterine dry weight ($P < 0.001$).

The butoestrol isomers were more effective inhibitors of uptake than the desoxyhexoestrol isomers (fig. 3). Ten µg *meso*-butoestrol significantly decreased oestradiol uptake by uterus and vagina. The *racemic* isomer

Table 2

Uterine dry weight after oestradiol and *threo*-desoxyhexoestrol administration. Every day for three days 10 µg *threo*-desoxyhexoestrol were injected in 0.1 ml oil 3 h before 0.003 µg oestradiol in 0.1 ml oil. The controls received oil or oil with only one of the test compounds. The animals were killed on the 4th day. Mean values ± s.e.m. are given. n = number of animals.

Treatment			Dry weight (mg)
0 h	3 h		
oil	oil	10	1.12 ± 0.038
threo-DH	oil	10	1.16 ± 0.036
oil	oestradiol	15	[illegible].34 ± 0.066
threo-DH	oestradiol	20	2.90 ± 0.160

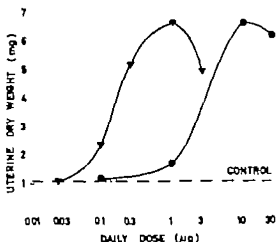


Fig. 4 Uterotrophic activity of vallestril ⓟ and TACE ⓡ. There were 5 animals/group. See also fig. 3
—▼—▼— vallestril —●—●— TACE

decreased uptake only at the 100 μg level The oestrogenic activity of the *racemic* form is also $\frac{1}{10}$ that of the *meso* form (fig. 2) Again, the uptake by liver and skeletal muscle was largely unaffected

Two important oestrogens, vallestril ⓟ and TACE ⓡ whose structure and geometric pattern are entirely different from those of the natural oestrogens and hexoestrol, were also studied. Vallestril is a potent oestrogen with a uterotrophic activity $\frac{1}{10}$ that of *meso*-hexoestrol, and TACE is less active (fig. 4). The uptake of oestradiol-^{3}H by uterus and vagina was effectively depressed by 100 μg vallestril. Ten μg vallestril slightly inhibits the uptake of oestradiol by the uterus ($P < 0.01$) but not by the vagina (fig. 5A) Three μg vallestril injected simultaneously with 0.01 μg oestradiol-^{3}H did not affect the uptake of radioactivity (fig. 5B) Higher doses could not be dissolved in 0 1 ml saline.

The oestrogenic triphenylethylene derivative TACE is known to have a long duration which is thought to depend on storage in body fat (cf. THOMPSON & WERNER 1953) One dose of this substance, 50 μg, was injected in 0 1 ml oil at various times before 0 01 μg oestradiol-^{3}H (table 3). Three hours after the injection of 50 μg TACE, 3 of 8 animals had a low uptake of oestradiol by the uterus and 1 of 8 a low uptake by the vagina the rest had, if anything, a higher uptake than the controls. Six hours after injection of 50 μg TACE, however all uteri and vaginae were blocked (cf table 3) Twelve hours after TACE injection the uptake of radioactivity had partly recovered The amount per organ approached the control level at 24 h (uterus) and 48 h (vagina)

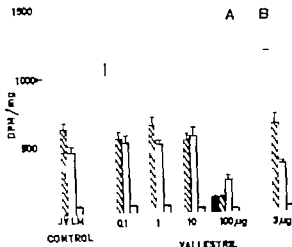


Fig. 5. Uptake of oestradiol-³H by various organs after vallestril ® administration.

A. The test compound was injected in oil 3 h before 0.01 µg oestradiol-³H in saline.

B. Simultaneously with the oestradiol-³H 3 µg vallestril were injected s.c. in 0.1 ml 10 % propylene glycol in saline. The animals were killed 1 h after the injection of radioactivity. There were 5–6 animals/group. Mean values are given vertical bars show one s.e.m.

Discussion

The uptake of radioactivity by the uterus and vagina of immature mice after the injection of oestradiol-³H was depressed by carrier oestradiol (TERENIUS 1965) A relationship between oestrogenic activity and inhibition of uptake was also found for the two 17-OH isomers of oestradiol We have now extended these findings to other oestrogens. The relation between ability to inhibit oestradiol uptake by the target organs and oestrogenicity is perhaps best studied by comparing compounds with similar structures but widely different biological activities. The hexoestrol series was most useful for this its members form many stereoisomers, and closely related analogues can be prepared.

The *meso* isomer of hexoestrol is about as uterotrophic in these mice as oestradiol The chemical structures of hexoestrol and oestradiol are markedly different, but there are similarities between their geometric patterns. CARLISLE & CROWFOOT (1941) found the X-ray photographs of *meso*-hexoestrol and the natural sex hormones to be closely related. UNGNADE & MORRISS (1947) found mixtures of oestradiol and *meso*-hexoestrol to be isomorphic by analyzing their melting-point behaviour Equal doses of *meso*-hexoestrol and oestradiol were about euqally effective as inhibitors of oestradiol uptake by uterus (cf table 4 column 3) and vagina. The two compounds thus seem to be also "bio-isosteric" in this property

Table 3

Uptake f oestradiol-³H by some rgans after prestimulation with TACE (8). At v rious tmes before the injection of 0 01 μg oestradiol-³H in 0.1 ml isotonic saline 50 μg TACE were injected in 0.1 ml live oil The animals were killed 1 h fter the injection of r dioactivity There ere 6–8 imals/group. Mean values ±s.e.m

Interval between TACE d oestrad l injectio	Uterus			Vagina		
	Wet weight (mg)	Dpm/organ	Dpm/mg wet weight	Wet weight (mg)	Dpm/o gn	Dpm/mg wet eight
Control (no TACE)	7 9	9390	1202 ± 73	7 4	3960	6-3 _ 52
3 h	8.7	870 – 15630[1]	116 – 1590[1]	6 6	610 – 6490[1]	107 – 1032[1]
6 h	10 0	1510	146 ± 16	7.5	1550	203 ± 33
12 h	11 3	5360	452 ± 46	7 3	3410	425 ± 51
4 h	15 3	9440	527 ± 37	13 6	3500	301 ± 54
48 h	32 0	12950	424 ± 51	23 9	4750	16 _ 19

[1] range.

Tissue	Control	Interval between TACE and oestradiol i jection				
		3 h	6 h	12 h	24 h	48 h
Liver Dpm/mg wet weight	463 50	33 _ 4	408 113	38 ± 23	465 _ 75	275 22
Muscle Dpm/mg wet weight	50 5 1	41 7 5	6 ± 5 1	27 ± 3 1	27 ± 2.2	_3 ± 5 4

The *racemic* hexoestrol isomer and the butoestrol isomers have physical properties closely similar to those of *meso*-hexoestrol and oestradiol, and the "passive" distribution patterns in the body are probably similar The desoxyhexoestrols are slightly less polar than the parent compounds. *Racemic* hexoestrol was about 100 times less uterotrophic than the *meso* form and the uptake inhibition capacity was also correspondingly lower This is certainly related to the fact that *racemic* hexoestrol in contrast to the *meso* form is not taken up by the target organs (unpublished observations)

In table 4 a comparison is made between the uterotrophic activities of the compounds and their capacities to inhibit oestradiol-³H uptake by the uterus. It must be borne in mind that the inhibition experiments were

Table 4

The relation between uterotrophic activity and uptake inhibition. The table is based on data from TERENIUS (1965) and results given in this communication. The inhibitors were administered in oil 3 h before the injection of oestradiol-³H in saline; animals were killed 1 h after the injection of the radioactive material. The percentage inhibition by a test compound of the uterine uptake of oestradiol-³H of the controls is given in column III. Doses causing a depression of uptake around 70 per cent are chosen. Thus in column IV the ratios

$$R = \frac{\text{Dose giving 70 \% inhibition}}{\text{Uterotrophic dose (from column II)}}$$

given. Values in brackets have been interpolated.

I Compound	II Uterotrophic dose (giving 50 % of maximum response) µg	III Inhibition of the uptake of oestradiol-³H Dose µg	 % inhibition	IV R
17 β-OH Oestradiol	0.005	0.1	45	(100)
		1	75	200
17 α-OH Oestradiol	0.4	10	72	5
meso-Hexoestrol	0.015	1	89	(40)
rac-Hexoestrol	1.5	30	68	20
erythro-Desoxyhexoestrol	5	100	44	>20
threo-Desoxyhexoestrol	40	100	0	–
meso-Butoestrol	3	10	68	3
rac-Butoestrol	50	100	75	2
Vallestril ®	0.15	100	90	(300)
TACE ®	3	50	87[1]	<18

[1] 6 h interval between TACE and oestradiol-³H injection.

made within a few hours, whereas the uterotrophic effects of the compounds were measured after stimulation during several days. Nevertheless, when the two stereoisomers of a compound are compared, it is evident that the more uterotrophic isomer also is a more effective inhibitor than the less uterotrophic. Further the dose ratio for 70 % uptake depression/50 % of maximum uterotrophic activity (table 4 column 4) is around 20 for many compounds. This relative constancy indicates that the inhibition is, in fact, competitive.

The butoestrol isomers had dose-response curves for uterotrophic activity that differed from those of the other compounds in being more shallow (fig. 2). Such oestrogens are called impeded (HUGGINS & JENSEN 1955). They were more effective uptake inhibitors, relative to their uterotrophic activities, than the other test compounds by a factor of ten (table 4). One might therefore expect them to be anti-oestrogenic in certain

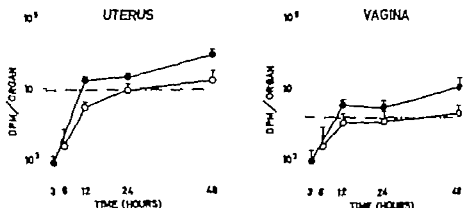


Fig. 6. Effect of pre-injection of oestradiol or TACE on the uptake of oestradiol-^{3}H by uterus and vagina. At various times before 0.01 μg oestradiol-^{3}H in saline 10 μg oestradiol or 50 μg TACE were injected s.c. in oil. The animals were killed 1 h after the injection of radioactivity. There were 5–8 animals/group. Mean values are given; vertical bars show three times s.e.m.

—●—●— oestradiol —○—○— TACE

The organ wet weights (mg) after prestimulation were oestradiol, 24 h: uterus 17.7, vagina 11.3; 48 h: uterus 31.6, vagina 29.4. TACE, 24 h: uterus 15.3, vagina 13.5; 48 h: uterus 32.8, vagina 23.9.

doses. It has indeed been found that at least the *meso* isomer of butoestrol is anti-oestrogenic to the vagina, but only after local application (EMMENS, COX & MARTIN 1962). Vallestril, on the other hand, was a weak uptake inhibitor.

Pre-injection of 50 μg TACE strongly depressed the uptake of oestradiol-^{3}H by uterus and vagina. Pre-injection of oestradiol or the hexoestrol isomers in oil solution caused strong inhibition of the uptake 3 h after injection. TACE in oil did not cause complete inhibition at that time, but only at 6 h after injection. This later appearance of the inhibition is most likely due to poor absorption of a highly hydrophobic compound. The depression of uptake 6 h after injection was even greater than that caused by an equi-uterotrophic amount of *meso*-hexoestrol or oestradiol 3 h after injection (table 4). The uptake of oestradiol-^{3}H by uterus and vagina various times after prestimulation with 10 μg oestradiol was investigated earlier (TERENIUS 1965). This amount given daily for 3 days is much more uterotrophic than similarly given 50 μg TACE. Single injections of these doses of the two compounds, however, give almost the same increase in the wet weight of uterus and vagina at 24 as well as at 48 h after injection. Fig. 6 compares the effects of prestimulation with the two oestrogens on the uptake of radioactivity per uterus and vagina. It appears that the initial depression of oestradiol-^{3}H uptake after TACE administration

returned to the control level almost at the same rate as after oestradiol pretreatment. The inhibition of the uterine uptake by TACE treatment was perhaps a little more protracted than that by oestradiol treatment. The strong initial depression and the rapid recovery of oestradiol-^{3}H uptake after TACE injection is surprising if one considers the long duration of its effect. The amount of radioactivity per organ 12 h after injection and later on was, however, higher after oestradiol pretreatment and increased above the control level. It cannot be stated with certainty whether this higher uptake depends on preferential stimulation of target cells by oestradiol or on prolonged inhibition by TACE or its metabolites.

At low doses 17 α-OH oestradiol, testosterone (TERENIUS 1965), the desoxyhexoestrols and *racemic* hexoestrol stimulated the uptake of oestradiol-^{3}H by uterus and vagina. *Threo*-desoxyhexoestrol also augmented the uterotrophic activity of oestradiol (table 2), which points to a relation between increased accumulation and final biological effect. After the work reported here had been completed BIALY, LAYNE & PINCUS (1965) reported that a urinary metabolite of norethynodrel[1]) injected together with oestrone resulted in potentiated uterotrophic activity. Certain doses of oestrone also give a small potentiation of the uptake of oestradiol-^{3}H (unpublished observation). Such potentiation may well have physiological importance.

Summary

Various synthetic oestrogens and analogues were administered systemically to immature mice. Tritiated oestradiol was given simultaneously or at various times later. The effect of the test compounds on the uptake of radioactivity by some organs was investigated. The preferential accumulation of oestradiol-^{3}H by uterus and vagina could be depressed by the test compounds to a degree that corresponded roughly to their oestrogenic activity. The stereoisomers of butoestrol were the most effective and vallestril the least effective of the inhibitors of uptake, relative to their oestrogenicity. The level of radioactivity in liver and muscle was essentially unaffected.

Acknowledgements

The author thanks Professor E. Bárány for criticism and Mrs. Carola Engström for competent technical assistance.

1) 17 Ethynyl-19-norandrost-4-ene 3 β, 10 β, 17 β-triol.

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A. L. Bacharach, M.A., F.R.I.C.

August 11th, 1891 – July 18th, 1966

In 1947 A. L. Bacharach wrote a letter to the editors of this journal full of compliments as to its scientific contents, but with less flattering remarks about the English in some of the articles, written with that touch of humour which was so characteristic of him. What was more important, however, he offered to correct the manuscripts into an "acceptable" language, and of course the editors accepted his suggestion with enthusiasm.

Since volume 4 and until now A. L. Bacharach has edited the English language of every article which has appeared in Acta Pharmacologica. He was a zealous guardian of the language allowing no laboratory slang or pseudo-scientific terms to sneak into the text, and no manuscript has been delivered to the printer without his corrections and critical remarks written in the green ink so well known by authors and editors of this Acta. Sometimes his corrections were slight, and their significance remained cryptic to the non English speaking reader, but most frequently they represented very obvious improvements. It also happened that phrases or paragraphs had the remark in the margin "What does he mean by

this?" and had to be completely rephrased Besides his editorial work he often gave the articles marks on attached slips, ranging from "One manuscript more in this terrible English and I will resign" to "This is one of the finest pieces of English to have come my way for several months" The first kind of slip was, of course, carefully removed before the authours saw their manuscripts again. The value of such a member of the editorial board cannot be overestimated

Bacharach's work for Acta Pharmacologica is much more admirable when his many other activities and interests are considered. He was president of the Nutrition Society chairman of the Food Group of Society of Chemical Industry vice president of the Royal Institute of Chemistry and Society of Chemical Industry and of Society of Analytic Chemistry

Moreover he was co-editor of many books, of which two have appeared recently "Evaluation of Drug Activities Pharmacometrics" with D R. Laurence (1964) and "The Physiology of Human Survival" with O C. Edholm (1965) In addition, he had a deep interest in music, and he also found time to edit books and articles on biographies of musical masters. Personally he was full of charm, and it was always a pleasure to meet him, be it here in Scandinavia when he was a visiting friend or lecturer or when one visited him in London.

The editorial board of Acta Pharmacologica et Toxicologica has suffered a great loss by the death of Alfred Bacharach. We shall miss him as a collaborator and a good friend.

Erik Jacobsen

Acta pharmacol et toxicol 1966 24, 103–111

From the Research Department f the Psychiatric Clinic, St Göran's Hospital, Stockholm, and the Institute of Zoophysiology University of Uppsala, Sweden.

Effect of Ethanol on Rat Liver
II. Number Size and Appearance of Mitochondria*

By

K. H. Kiessling and L. Pilström

(Received October 26, 1965)

We have previously reported that the liver from rats receiving ethanol over a long period contained numerous mitochondria of altered shape and size compared with those of controls (KIESSLING & TOBÉ 1964 KIESSLING & PILSTRÖM 1966). The commonest was a greatly enlarged type. Other deviations from the controls were a reduced oxidation rate of various mitochondrial substrates (KIESSLING & PILSTRÖM 1966 KIESSLING & TILANDER 1961), a decreased ratio between the DNP and the Mg^{2+} stimulated ATP-ases (KIESSLING & TILANDER 1961) and a decreased thiamine-diphosphate level (KIESSLING & TILANDER 1960)

This report deals with the degree of enlargement of liver mitochondria, their matrix and cristae and the quantity of mitochondria, all as a consequence of ethanol treatment. It also deals with the question of how soon the mitochondrial size is normalized after ethanol has been withdrawn.

Experimental

Treatment of animals

Wistar rats from this laboratory' stock were used. The males f each litter were divided into two groups, one being used for ethanol treatment and the other as controls, drinking water and sugar solution isocaloric with the ethanol consumed by the ethanol group. Both groups had free access to adequate solid food. The ethanol used was 15% (/v) solution. Each animal was kept in separate cage and had free access to either water (controls) or ethanol (ethanol treated group). The animals were treated n this way for eight months before liver samples wer taken. The mean fluid intak during this time was

*) Part I of this nvestigation is n *Quart. J Stud. Alc* 1966, 27 in press.

8*

about the same in both groups (bout 27 ml per day) During the whole treatment period the rats drinking ethanol grew more slowly than their controls (mean weight after 60 days: ethanol treated rats 250 g, controls 325 g after 210 days 300 g and 380 g, respectively).

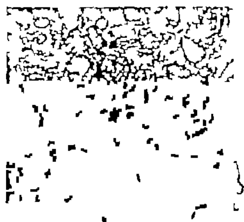
Electron microscopy

Small pieces of liver were removed from the peripheral part of a liver lobe with the rat under ether narcosis. The pieces were fi ed in an OsO_4 solution for two hours, dehydrated i ethanol and embedded in Epon. The electron microscope used was an Akashi Tronscope Model TRS-50 E1

A necessary pre-requisit when comparing the size of mitochondria from the alcohol-treated animals with that f controls by electron microscopy is that the photographs should be taken completely at rand m. No possibility must exist for conscious or unconscious selection of motives that support a hypothesis. In order to avoid this the person taking the micrographs did not know from which animal the sample originated. Secondly the motives were chosen t such low enlargement (1000 ×) that there was no possibility of deciding whether or not the mitochondria were larger tha normal. Not until then was the wanted enlargement (3000 ×) made.

On th micr graphs (final enlargement 9000 ×), every mitochondrial profile was measured, i dependent of size.

Each animal was represented by si grids from t o liver pieces and from three levels of each piece.



F g 1 Electro micrograph of liver mitochondria from rat receiving ethanol f eight months

Greatly enlarged malformed mitochondria with disorganized crista re mred th those of normal ppearance. I th greatly enlarged mitochondria the matrix is not or only slightly clearer than normal. Enlargement 7,200 ×

The photograph has been selected in order to show various types of mitochondrial deviation observed connection with prolonged ethanol treatment. Although those th a more r less narrowed middle section occu the most frequently bserved deviations are large oval mitochondria with disorganized cristae.

From those micrographs the longest and the shortest axes of each mitochondrion were measured and the cross-sectional areas of the mitochondrial profiles were calculated as previously described (KIESSLING *et al.* 1966). In fig. 1 are seen mitochondria of various shapes to be found in rat liver after prolonged ethanol treatment. The undoubtedly commonest deviation from the normal mitochondrion is the one seen on the picture as a large oval, whereas deviations in the shape of mitochondria with a narrow middle section are relatively rare. Thus this method of calculating the area of the mitochondrial profiles can cause only minor errors.

A material obtained at random for measuring the length of the cristae was obtained by letting some one unacquainted with the problem pick out several enlarged liver mitochondria from the alcohol-treated rats and a corresponding number from the controls. The micrographs were then enlarged 50,000 times, which made it possible to measure the length of the cristae.

In using the electron microscope technique for quantitative studies of this kind, one source of error may be the compression of subcellular particles during the slicing procedure. A thorough examination of the photographs used for mitochondrial shape in relation to the sectional direction excluded this factor as of no importance in calculating the mitochondrial size.

Results

Mean size of mitochondria

The size of liver mitochondrial profiles was examined by means of electron micrographs from 15 control and 22 alcohol-drinking rats (eight months on ethanol). All liver pieces were taken from the periphery of a liver lobe. However, no differences of variation in mitochondrial size was found between peripheral and central parts of a lobe either in alcohol treated rats or in water-drinking controls (KIESSLING & PILSTRÖM 1966). At least 300 mitochondrial profiles were measured from two liver pieces of each animal. From these figures we calculated the mean areas for the liver mitochondrial profiles from the two experimental groups, the con-

Table 1

The size of mitochondrial profiles in liver from rats consuming alcohol and from controls.

	Control	Alcohol
Number of mitochondria	6119	8548
Number of animals	15	22
Total mitochondrial area (μ^2)	2442.04	4949.62
Mean area (μ^2)	0.399	0.579
Standard deviation (μ^2)	0.056	0.105

t-value = 6.74 d.f. 35 $P < 5 \times 10^{-4}$

trols (drinking water) and the ethanol-consuming rats (table 1). The P value of less than 5×10^{-4} shows a significant difference in the mitochondrial area between the two groups. This means that the mean area of the mitochondrial profiles from the alcohol treated animals is nearly 50% larger than that from the controls.

If we rotate the ellipse representing the area of a mitochondrial profile around its longest axis, we obtain a body that we assume roughly to represent the volume of the mitochondrion. We applied this way of calculating the volume to 200 mitochondria from an alcohol-treated rat and its control. The ratio between the mean areas of the liver mitochondrial profiles from the alcohol treated animal and from the control was 1.54. The ratio between the mean volumes was 1.93 indicating that, on an average, the liver mitochondria from the alcohol treated animals are nearly twice as big as those from controls.

Cristae and matrix

The length of the cristae was measured in a number of large liver mitochondria from five alcohol treated rats and in mitochondria from as many controls. The results in table 2 show that the length of the cristae in the enlarged mitochondria is only about half that of the cristae from controls.

We know of no reliable method of measuring the density of the mitochondrial matrix. Our impression, from the electron microscopic examination of a large number of normal and enlarged mitochondria is, however that most pathologically enlarged mitochondria contain a matrix that is not at all or only slightly diluted compared with normal ones, indicating no water imbibition (fig. 1)

Table 2

Cristae in enlarged mitochondria from alcohol-treated rats and in mitochondria from controls.

	Control	Alcohol
Number of mitochondria	39	49
Mean length of the combined cristae per mitochondrion (μ)	1.681	0.788
Cristae/mitochondrial area (μ/μ^2)	2.60	0.63
Standard deviation (μ/μ^2)	0.898	0.426

t-value = 12.63 d.f. 86 $P < 5 \times 10^{-4}$

Table 3

The number of mitochondria in liver from rats consuming alcohol and from controls.

	Control	Alcohol
Number of mitochondria/mm² ($\bar{X}$)	2.52×10^5	2.35×10^5
Standard deviation	0.46×10^5	0.48×10^5

t-value = 1.08 d.f. 35 P ≈ 0.30

Number of mitochondria

From electron micrographs of the livers of 15 control and 22 alcohol treated rats the tissue area was measured on which more than 9000 and 15 000 mitochondria respectively were scattered (table 3). About the same tissue area was investigated from each animal. The results are expressed as the number of mitochondria per mm² of liver tissue. No significant difference was found between the two groups.

Return to normal size

Five rats that had consumed ethanol for at least eight months were used. Liver biopsies were taken when the rats were still on alcohol and the mitochondrial profiles were measured on electron micrographs. About four and seven months after the withdrawal of alcohol new biopsy specimens were taken from the same rats, and the mitochondrial profiles were again measured. The results are seen in fig. 2. The points show the mean values for the five animals. Only in one of the animals was a complete return to normal size obtained within four months.

Discussion

Rat liver cells have been reported to have anything from 500 to 2500 mitochondria, with an average of about 800 per cell. Observations on changes in the number of mitochondria in relation to variations in the physiological state of the tissue occasionally occur in the literature. Thus HOWATSON & HAM (1955) and DAVID (1959) found a low number of mitochondria in embryonic rat liver. The transition from pre- to postnatal life involves an increase in the number of mitochondria, as well as in the number of cristae per mitochondrion (DAVID 1964). In muscle there is an obvious relationship between tissue activity and the number of

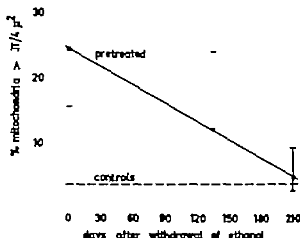


Fig. 2. The normalization of mitochondrial size after withdrawal of ethanol.
The rats were given an ethanol solution (15 % v/v) for eight months, after which ethanol was exchanged for water. The dots are mean values from five animals. The highest and lowest values for each occasion are also given.

mitochondria, including their concentration of cristae. In highly active striated muscle (red thoracic flight muscle and heart) mitochondria are numerous and cristae densely packed (BENNET *et al.* 1953, HOWATSON 1956, MOORE *et al.* 1957, LINDNER 1957), whereas in white muscle and in smooth muscle mitochondria are less numerous and cristae less densely packed (BENNET *et al.* 1953), MARK 1956).

Changes in mitochondrial number have also been observed in pathological conditions. DAVID (1964) found a reduced number of animal liver mitochondria in serious nutritional deficiency. In severe hypermetabolism with a defective mitochondrial respiration control LUFT *et al.* (1962) found an increased number of mitochondria in skeletal muscle. It is conceivable that the increase there was a compensatory mechanism to overcome the handicap imposed by the decreased efficiency of the mitochondria.

These examples may indicate a connection between tissue activity and number of mitochondria, as well as density of cristae per mitochondrion.

After prolonged ethanol consumption by rats we observed in the liver an obvious increase in mitochondrial size and also a shortening of the cristae. These structural changes are probably connected with the decreasing metabolic activity previously reported (KIESSLING & PILSTRÖM 1966, KIESSLING & TILANDER 1961). ROUILLER (1960) distinguishes between two types of mitochondrial enlargement, one caused by water imbibition attended by a diluted matrix and the other by a concentration of

substances attended by a condensation of the matrix. In both types the cristae may shorten or disappear The phenomena are also discussed by DAVID (1964) Electron microscope observations, exemplified by fig 1 indicated that in our examples the enlargement was only partly if at all, caused by water imbibition. Only in a few of the enlarged mitochondria was the matrix clearer than in normal mitochondria. A matrix denser than normal hardly ever occurred in the enlarged rat-liver mitochondria after ethanol treatment of the animals.

However as is shown in table 3 no increase in the number of mitochondria could be observed. Thus, although ethanol administered for a long time causes mitochondrial malformations, including shortening of the cristae and a decreased capacity to oxidize mitochondrial substrates, we could not establish any tendency to compensate for this reduced capacity with an increased number of liver mitochondria.

The disappearance of pathologically shaped mitochondria after the withdrawel of ethanol is extremely slow (fig. 2) and exceeds the half-life of liver mitochondria, that is, about 10 days (FLETCHER *et al* 1961) by more than 10 times. This slow return to mitochondria of normal shape is difficult to explain

The bulk of the experimental evidence on the biogenesis of mitochondria favours the idea that pre-existing mitochondria may grow and divide They contain their own DNA (CHEVREMONT *et al* 1960 NASS *et al* 1965) and RNA (NEUBERT *et al* 1965), which gives them, at least partly the possibility of synthesizing their own proteins. At present it seems unlikely that ethanol could have influenced these processes to produce the long-lasting mitochondrial abnormality

If however the synthesis of biological membranes occurs by the mechanism summarized by LEHNINGER (1964), the membranous structure of the mitochondrion may possibly have the enzymatic capacity to produce new membranes in such a manner that the preexisting membrane serves as a template for its own assembly from specific proteins and lipids. A change in the membrane composition caused by ethanol may therefore survive the single mitochondrion several times over

A study of the membrane composition of the malformed mitochondria is in progress.

Summary

Liver mitochondria from rats fed ethanol for eight months have been studied by means of electron microscopy The mean area of the mitochondrial profiles, calculated from electron micrographs, is about 1.5 times that of mitochondria from controls. The cristae of the enlarged mitochondria are much shortened, on an average to half that of normal liver

mitochondria. The density of the matrix seems to be not at all or only slightly diluted compared with that of normal mitochondria. No significant difference in the number of mitochondria could be found between liver pieces from rats drinking ethanol and liver pieces from their controls.

The return to normal size after ethanol is withdrawn is slow (100–200 days) A possible explanation of this is briefly discussed.

Acknowledgements.

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We are indebted to Prof C. H Alström for placing the necessary facilities at our disposal to Prof P E. Lindahl for giving us the opportunity to use the electron microscope to Miss Maj-Britt Jansson, Miss Helga Möbius and Miss Ann-Sofie Wallin for skillful technical assistance

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Acta pharmacol et toxicol 1966, 24 112–120

From the Department of Pharmacology University of Göteborg, Göteborg, Sweden.

The Interference of Tetrabenazine, Benzquinamide and Prenylamine with the Action of Reserpine

By

Arvid Carlsson and Margit Lindqvist

(Received October 30, 1965)

The specific mechanism dependent on ATP Mg^{++} for incorporating monoamines into the storage granules of the adrenal medulla and adrenergic nerves is selectively blocked by compounds such as reserpine, tetrabenazine and prenylamine (segontin ®) (CARLSSON, HILLARP & WALDECK 1963 EULER & LISHAJKO 1965 LUNDBORG 1966) In the initial stage the block induced by reserpine is competitive (CARLSSON, HILLARP & WALDECK 1963 JONASON ROSENGREN & WALDECK 1963) and can thus be partially prevented by another agent with the same point of attack but shorter duration of action. QUINN SHORE & BRODIE (1959) were thus able to demonstrate that rabbits treated with tetrabenazine (50 mg/kg) 15 minutes before reserpine (1 mg/kg) responded in behaviour as though they had received only tetrabenazine after 24 hours the animals had recovered, whereas the rabbits treated with reserpine alone were still heavily sedated Also the duration of 5-hydroxytryptamine (5-HT) depletion in brain was found to be shortened by tetrabenazine pretreatment. However no statistically significant effect on noradrenaline (NA) depletion was detected.

WEISSMAN & FINGER (1962) have reported that benzquinamide (50 mg/kg i.p) a benzoquinolizine closely related to tetrabenazine, caused mild sedation and a decrease in both 5-HT and NA of rat brain At lower doses that did not affect brain amine levels this drug caused disruption of conditioned avoidance reactions without overt sedation. *In vitro*, benzquinamide at relatively high concentration (5×10^{-4} M) has been found to cause blockade of the ATP Mg^{++} dependent uptake by adrenal medullary granules (LUNDBORG 1965 personal communication)

In this paper the ability of tetrabenazine, benzquinamide and prenylamine to interfere with the action of reserpine on brain and heart monoamine levels is reported [1])

Methods

The experiments were performed on white female mice, weighing about 20 g, and on white rabbits of both sexes, weighing about 2 kg. Drugs were injected as described under Results. The animals were killed at various intervals after the injections. Unless otherwise stated, the experiments on mice were performed at a constant temperature of 29° and on rabbits at room temperature (23–24°).

Amine determinations were performed on brain, heart and adrenals, adrenaline and NA by the method of BERTLER, CARLSSON & ROSENGREN (1958), dopamine (DA) by the method of CARLSSON & WALDECK (1958) with the modifications described by CARLSSON & LINDQVIST (1962), 5-HT by the method of BERTLER (1961), except that the perchloric acid residue was re-extracted once with 0.4 N perchloric acid.

Results

Mice were injected intraperitoneally with tetrabenazine 100 mg/kg and 30 minutes later with reserpine 1 mg/kg. Control mice received tetrabenazine or reserpine alone. Analogous experiments were performed with benzquinamide 100 mg/kg, and reserpine 1 mg/kg after 30 minutes, and then two reinjections of benzquinamide (50 mg/kg each) 30 and 60 minutes after the reserpine injection. Prenylamine was given as two intravenous injections of 10 mg/kg each, 30 and 5 minutes before reserpine (1 mg/kg i.p.). In the prenylamine experiments the animals were kept at room temperature. The mice were beheaded 24 hours after the first injection.

A few minutes after the injection of tetrabenazaine or benzquinamide alone the mice became heavily sedated and could hardly be activated, but 2–3 hours later they began to recover but the animals that had received reserpine as well as tetrabenazine or benzquinamide were still heavily sedated. The sedation of these mice was more pronounced than after reserpine alone. The animals had recovered fairly well 6 to 8 hours after the injection of tetrabenazine or benzquinamide alone, and at 24 hours they showed a normal gross behaviour. At 24 hours the reserpine mice were still sedated, whereas the mice that had received tetrabenazine plus reserpine appeared almost normal. The animals that had received benzquinamide with reserpine were more sedated than those treated with reserpine alone. Prenylamine given alone caused little or no sedation. It protected the animals from the sedative action of reserpine to a certain

[1]) A preliminary communication of these result was made at the International symposium on mechanisms of release of biogenic amines, Stockholm, Febr. 21–24, 1965.

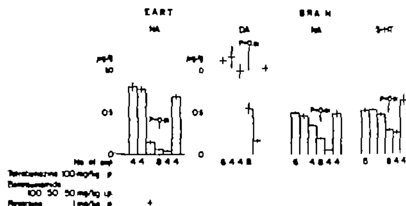


Fig. 1 Effect of tetrabenazine or benzquinamide pretreatment on monoamine depletion of mouse brai and heart by reserpine.

Tetrabenazine or benzquinamide was given 30 minutes before reserpine. Two reinjections of benzquinamide were made 30 and 60 minutes after reserpine. The nimals were killed 23 5 hours after reserpine.

NA = noradrenaline. DA = dopamine. 5-HT = 5-hydroxytryptamine.

Each analysis was performed on 6 pooled organs.

Vertical lines t top of columns indicate standard errors of the means. Vertical bars bove columns indicate differences necessary for p = 0.01 For brain monoamines this difference has been calculated for each amine by analysis of variance and refers to one group of 4 nimals and one group of 6 animals. It is seen that the effect of tetrabenazine and benzquinamide pretreatment is significant at the 1 per cent level, except for 5-HT after benzquinamide pretreatment. For heart NA, the statistical significance test was performed on the two groups treated with tetrabenazine plus reserpine or reserpine alone.

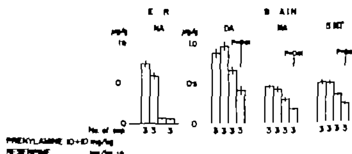


Fig. 2 Effect of prenylamine pretreatment on monoamine depletion f mouse brain and heart by reserpine.

Prenylamine was given 30 and 5 minutes before reserpine. The animals were killed 23.5 hours after reserpine

NA = radrenaline. DA = dopamine. 5-HT = 5-hydroxytryptamine.

Each analysis was perf rmed on 6 pooled organs.

Vertical lines at top of columns indicate standard errors of the means. Vertical bars above columns indicat the difference between two groups of 3 animals necessary for p = 0.01 calculated for each amine by analysis of variance. For all three brain monoamines, the effect of prenylamine pretreatment is significant at a level of bout 0.01

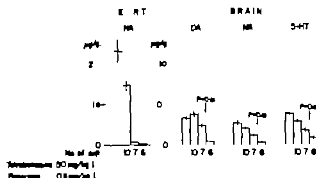


Fig. 3 Effect of tetrabenazine pretreatment on monoamine depletion of rabbit brain and heart by reserpine.

Tetrabenazine was given 15 minutes bef re reserpine. The animals were killed 23½ hours after reserpine.

NA = noradrenaline. DA = dopamine. 5-HT = 5-hydroxytryptamine.

Analyses were performed on the organs of individual rabbits.

Vertical lines at top of columns indicate standard errors of the means. Vertical lines above columns indicate the difference between two groups of 7 and 6 animals necessary for $p = 0.01$ calculated for each amine by analysis of variance. For brai DA nd NA the effect of tetrabenazine pretreatment is significant at the 1 per cent level, for brain 5-HT the corresponding difference is significant at the 2.5 per cent level.

extent, but seemed to be less efficient than tetrabenazine at this in the doses employed. Higher doses of prenylamine could not be used because of its toxicity

Treatment with tetrabenazine partly but significantly prevented the reserpine induced decrease in tissue amine levels (fig. 1). The brain levels of NA, DA and 5-HT in these pretreated mice were 70 88 and 92% of normal, respectively compared to 40, 49 and 56% respectively after reserpine alone. The differences are statistically significant (see legend to fig. 1) Pretreatment with benzquinamide, however caused a decrease in brain NA and DA to 12 and 15% respectively of the normal values, which is significantly lower than after reserpine alone. The 5-HT levels appeared to show the same tendency but the difference was not statisti cally significant (fig. 1) Prenylamine partially protected against reserpine in its effect on brain DA NA and 5-HT (fig. 2) the differences being statistically significant.

In the heart similar effects to those in brain may have been produced, but were much less pronounced. In the control groups with tetrabenazine, benzquinamide or prenylamine alone the brain and heart amine values obtained at the interval studied (24 hours) did not differ significantly from normal.

Analogous experiments were performed on rabbits which received

Table 1

Effect of tetrabenazine pretreatment on monoamine depletion of rabbit brain and heart by reserpine.

	Brain			Heart
	NA	DA	5-HT	NA
Tetrabenazine 50 mg/kg i.v. + Reserpine 1 mg/kg i.v.	0.07 ±0.027 (4)	0.18 ±0.046 (4)	0.09 ±0.020 (4)	0.02 ±0.011 (4)
Reserpine 1 mg/kg i.	0.01 ±0.003 (3)	0.06 ±0.005 (3)	0.03 ±0.007 (3)	0.01 ±0.006 (3)
Tetrabenazine 50 mg/kg i.v.	0.[illegible]1 ±0.014 (10)	0.38 ±0.031 (10)	0.29 ±0.025 (10)	1.46 ±0.129 (10)
Normal	0.27 ±0.031 (4)	0.33 ±0.020 (4)	0.38 ±0.017 (4)	2.30 ±0.277 (4)

Tetrabenazine was given 15 minutes before reserpine. The animals were killed 23½ hours after reserpine.

The values are means ± standard errors of the means, expressed as µg/g tissue. Figures in brackets indicate number of animals.

tetrabenazine 50 mg/kg intravenously and reserpine 0.5 mg/kg intravenously 15 minutes later. The animals were killed by air embolism 24 hours after the injection of tetrabenazine. Control rabbits received tetrabenazine or reserpine alone. The symptoms of the rabbits were similar to those of the mice. After tetrabenazine alone, and after tetrabenazine with reserpine, strong reserpine like effects set in rapidly, lasted 2–3 hours and were then succeded by recovery. These animals showed normal gross behaviour after 24 hours when the animals treated with reserpine alone still showed typical reserpine symptoms.

Fig. 3 shows the mean levels of DA, NA and 5-HT in the rabbits brain and of NA in the heart. A clearcut and statistically significant protective effect of tetrabenazine against reserpine action was seen on all three brain amines (see legend to fig. 3). The heart NA levels were low in both groups.

In some experiments a larger dose of reserpine (1 mg/kg intravenously) was used (table 1). The same tendency appeared, but the protective action of tetrabenazine against this dose of reserpine was slight.

In the adrenals of mice and rabbits no significant protective action of tetrabenazine was found (results not shown).

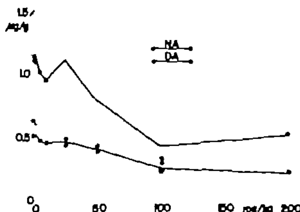


Fig. 4. Noradrenaline (NA) and dopamine (DA) in mouse brain 25 minutes after various intraperitoneal doses of benzquinamide. Each analysis was performed on 3 to 5 pooled brains

The unexpected results with benzquinamide prompted us to study the effects of this drug on amine levels

Various doses of benzquinamide (5 to 200 mg/kg) were given intra peritoneally to mice, and DA and NA were determined in the brain 25 minutes after the injection. A 50 / decrease in these amines required a dose of about 100 mg/kg (fig. 4)

After the 100 mg/kg dose DA and NA in brain showed a decrease, with the lowest values at about 25 minutes and a return to normal values within 6 hours 5-HT showed only slight deviations from the normal value (fig. 5) In these two experiments the mice were kept at room temperature.



Fig. 5 Noradrenaline (NA), dopamine (DA) and 5-hydroxytryptamine (5-HT) in mouse brain at different intervals after benzquinamide, 100 mg/kg i.p. Each analysis was performed on 3 to 6 pooled brains.

Discussion

The results of this investigation are partly in agreement with those of QUINN *et al* (1959) tetrabenazine was found to protect rabbits and mice against the action of reserpine on behaviour as well as on brain 5-HT In our work a somewhat lower dose of reserpine had to be used in order to obtain a clearcut protective effect of tetrabenazine, possibly owing to a difference in sensitivity of the animals to reserpine QUINN *et al* observed no significant protective action of tetrabenazine on brain NA. In our study the protective effect of tetrabenazine appeared to be about the same on brain NA, DA and 5-HT The reason why QUINN *et al.* found no significant effect on brain NA may be that the experimental group in question consisted of only two rabbits. Our investigation lends additional support to the conclusion of QUINN *et al* that tetrabenazine and reserpine have identical points of attack.

The other benzoquinolizine investigated, i e. benzquinamide, was found to have an unexpected effect, namely to enhance the behavioural and biochemical effects of reserpine The mechanism of this effect is obscure. Like tetrabenazine, benzquinamide causes a transient decrease in brain catecholamine levels, but it is uncertain if the two drugs act by the same mechanism We have observed that they act differently after monoamine oxidase inhibition After pretreatment with nialamide, 100 mg/kg, given intraperitoneally to mice, tetrabenazine causes excitation (cf PLETSCHER 1962) whereas benzquinamide causes sedation under these conditions also (cf WEISSMAN & FINGER 1962) It therefore seems probable that the two drugs act by different mechanisms. The enhancement of the action of reserpine by benzquinamide may be related to its unknown mode of action in the brain, but other possibilities exist. For example, benzquinamide, which like reserpine is an ester may compete with reserpine for metabolic pathways in the liver and thus enhance and prolong its actions.

Like tetrabenazine, prenylamine partly protected against the actions of reserpine on behaviour and on brain monoamine levels. It therefore seems likely that the site of action of prenylamine on the ATP Mg^{++} dependent storage mechanism of the amine granules is identical with that of reserpine and tetrabenazine. One might ask why prenylamine does not cause sedation and other typical reserpine actions. No answer to this question can be given at the present time. It should be noted, however that the protective action of prenylamine in the doses employed was relatively weak Similarly prenylamine does not seem to be capable of causing the same marked degree of monoamine depletion as the other two drugs. It thus seems possible that the action of prenylamine is not powerful enough to cause interference with monoamine transmission

mechanisms. Further the possible occurrence of different monoamine pools should be considered Evidence is now accumulating that the blocking action of drugs such as reserpine and tetrabenazine on monoamine transmission is primarily due to depletion of a small functionally essential pool rather than of the amine store as a whole. It may be that the ability of prenylamine to deplete this pool is less than that of tetrabenazine and reserpine In this connection it should be noted that in man treatment for 5 days with prenylamine results in certain reserpine-like actions, *e.g* bradycardia and reduced responsiveness to the pressor action of tyramine (KUSCHKE *et al* 1964 & 1965) This suggests that prolonged treatment with prenylamine will ultimately result in depletion of the small functionally essential pool, at least in certain monoamine-containing nerve terminals.

Summary

Mice and rabbits were treated with tetrabenazine, benzquinamide or prenylamine shortly before an injection of reserpine After 24 hours the animals were killed, and the brains and hearts were analysed for monoamines Tetrabenazine and prenylamine partially protected the animals against the action of reserpine on gross behaviour and brain monoamine levels (dopamine, noradrenaline and 5-HT), indicating that these two drugs compete with reserpine for the same site of attack in the amine storage granules. Benzquinamide enhanced the effects of reserpine on behaviour and on brain catecholamine levels. The mechanism of this effect is obscure.

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From H. Lundbeck & Co. A/S (Departments of Synthetic Chemistry (1) and Pharmacology (2)), Ottiliavej 7, Copenhagen – Valby

Pharmacological Studies of a New Series of Bicyclic Thymoleptics

By

P. V. Petersen[1], N. Lassen[1], V. Hansen[1], T. Huld[1], J. Hjortkjær[1], J. Holmblad[1], I. Møller Nielsen[2], M. Nymark[2], V. Pedersen[2], A. Jørgensen[2] and W. Hougs[2]

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Antidepressants of the thymoleptic group are derived from the phenothiazine and thiaxanthene types of neuroleptics on replacing the S-bridge between the two benzene rings by a carbon-bridge. Thus the currently known thymoleptics are all derivatives with two benzene rings condensed to a tricyclic ringstructure through a central alicyclic or heterocyclic ring.

Pharmacologically the thymoleptic activity of these compounds has been related to potentiation of catecholamines and to antagonism against reserpine induced sedation and ptosis. Further all thymoleptics known today have strong anticholinergic activity. In fact, the central anticholinergic properties have by some investigators been considered the key to antidepressant action, though others tend to relate this effect to a potentiation of adrenergic mechanisms in the CNS.

It is important to realize that the close chemical relationship between the tricyclic thymoleptics and the corresponding neuroleptics is reflected also in some degree of functional relationship. Thymoleptics seem to possess dual activity: thus the thymoleptic properties (potentiation of catecholamines and reserpine antagonism), which are clearly seen at low dosage, tend to reverse into neuroleptic properties (antagonism towards catecholamines and synergism with reserpine) at high dosage.

It might be expected that, by studying compounds with more remote structural relationship to known neuroleptics, a substance possessing only the thymoleptic properties could be found.

Proceeding along these lines and employing noradrenaline potentiation and reserpine antagonism as criteria for thymoleptic activity, we have

synthesized and studied a series of compounds, in which the tricyclic ringstructure has been replaced by a bicyclic structure. Although the fundamental structure is a bicyclic ring system with only one benzene ring, it will nevertheless be shown that the highest activity is obtained with the compounds that, owing to an aromatic substitution in the bicyclic system show greatest resemblance to the tricyclic thymoleptics.

Materials and Methods

A. *Chemical*

The compounds under study were derivatives of one of the chemical structures shown in fig. 1

The activities of these substances were compared with those of a number of known or new tricyclic compounds (cf. table 4).

Detailed descriptions of the configurations of all compounds are given in tables 1 to 4, column 2. Each compound was prepared as the most convenient water-soluble salt, named in column 4 of the tables. When no suitable salt was obtainable, solutions of the bases were prepared by adding hydrochloric acid. The results shown in columns 5 to 10 of the tables are based on the substances as weighed.

B. *Pharmacological*

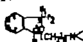
Acute toxicity was determined by intravenous injection into male mice (NMRI), 18–22 g, kept in single cages at 23°. The LD50 values, based on 3 to 4 dose levels (5 mice per group) were determined by the method of MILLER & TAINTER (1944).

Reserpine antagonism. 1) *A preliminary estimation* of antireserpine activity was made by injecting the test compound intraperitoneally at the dose levels of 10 and 50 mg/kg to two groups of 3 male mice each. The animals were then observed for signs of CNS activity (sedation or stimulation) for one hour. Then a standard dose of reserpine 5 mg/kg was given intravenously. Observation of the occurrence and degree of ptosis and presence or absence of the typical reserpine syndrome was made during the next hour. In each experiment two untreated control groups were included, of which one received reserpine after the first hour. The degree of antagonism against ptosis and immobility was roughly estimated on a scale ranging from 0 to + + +. The observer was unaware of the identity of the experimental groups until after the experiment. This test was used only as a quick guide to the compounds that be most suitable for the more detailed study.

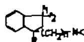
2) *Prevention of reserpine-induced ptosis.* Unfasted male mice (NMRI) in the weight range of 18–25 g were treated with test drug. A group of at least 5 animals per dose per drug was formed and placed in a cage. An untreated group served as control. The injections of test compounds were done by some one different from the observer, the latter not being informed about the identity of the groups until after scoring. After half an hour had elapsed, all animals were given reserpine (serpasil ®, 2.0 mg/kg) and returned to their originally assigned cages. One hour after administration of the challenging dose of reserpine the cages were tilted up and down a few times, and 30 seconds later the animals were scored for the degree of ptosis.

The scoring system adopted by RUBIN *et al.* (1957) was used: 4 = complete, 3 = ¾, 2 = ½, 1 = ¼ closure of the eyelids. Normal opening was scored zero. If different degrees of ptosis in the two eyes of an animal were observed, the average score was used. All injections were given intraperitoneally and the volume injected was 0.1 ml/10 g. The activity of each

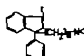
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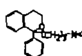
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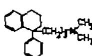
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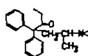
IV Isochromanes



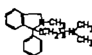
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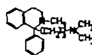
VI Methadone deriv.



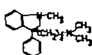
VII Isoindolines



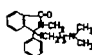
VIII Tetrahydroisoquinolines



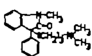
IX Indolines



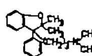
X Phthalimidines



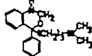
XI Oxindoles



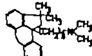
XII Dihydrobenzofuranes



XIII Dihydrobenzoxazines



XIV

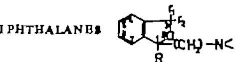


test drug was expressed as the dose (mg/kg) that reduced the ptotic score to half that of control mice (ED_{50})

Noradrenaline (NA) potentiating effect was estimated in the pithed rat preparation of SHIPLEY & TILDEN (1947) as described earlier (MØLLER NIELSEN & NEUHOLD 1959). A small submaximally active dose, i.e. 0.05 µg/kg i.v. of NA giving pressor response of 10–20 mm Hg was used as standard. After i.v. injection of the test drug, the augmentation of the NA response was calculated as percentage of the initial value. When 1 mg/kg of the test drug yielded less than 50% potentiation and the antireserpine effect was found weak, usually no further doses were tested, and the result (tables 1 to 4) was given as >1000 (weak effect) or ≫1000 (extremely weak effect). In other experiments at least 3 dose levels were tested,

Table 1

I PHTHALANES



1	2	3	4	5	6	7	8	9	10
Serial / code no. / no. $R_1 = -CH_3$ $n = 3$	R	Amino-group'	Salt	Melting point °C	LD50 mg/kg iv	Reserpine Antagon. Prelim.	Ptosis ED50 mg/kg ip	Reversal mg/kg ip	NA Potent. ED50 mcg/kg iv
1/Lu 3-009	C_6H_5-	D	HCl	172–73	67	+++	1	>64	450
2/Lu 3-010		M	HCl	190–91	71	+++	0 4	>64	10
3/Lu 3-076	p-Cl-C_6H_4-	D	base	bp 1 mm 173–75		+	>8		>1000
4/Lu 3-075		M	HCl	178–79	75	+	~3		
5/Lu 3-020	p-CH_3-C_6H_4-	D	HCl	180–81	48	++(+)	1–2		2500
6/Lu 3-001	m-CF_3-C_6H_4-	D	HCl	162–64	47	+++	3		1650
7/Lu 3-002		M	H_2SO_4	157–60	66	+++	1		160
8/Lu 3-053	cyclohexyl	D	oxalate	100–15	56	+	>8		
9/Lu 3-028	H	D	oxalate	116–18	76	+	21		520
10/Lu 3-071		M	oxalate	170–72	67	+	3–4		~1000
11/Lu 3-034	OH-	D	oxalate	154–58	173	+	>8		>1000
12/Lu 3-019	(6-Cl) C_6H_5-	D	HCl	196–98	52	++(+)	~2		>1000

R = C_6H_5 = 3	r_1	r								
13/Lu 3-035	CH_3	H	D	oxalate	115–16	67	+++	~3		1350
14/Lu 3-051			M	oxalate	190–31	72	+++	~2	>64	66
15/Lu 3-018	H	H	D	HCl	176–77	37	+++	~8	>64	2500
16/Lu 3-039			M	HCl	116–18	67	++(+)	>8		270
17/Lu 3-037	C_2H_5	$C\,H_5$	D	HCl	151–5	57	+	>8		>1000
18/Lu 3-044			M	oxalate	188–90		++(+)	~8		400
19/Lu 3-033	C_2H_5	H	D	HCl	160–63	48	+	~1**	2	~8000
20/Lu 3-029	CH_3	OH	D	base	120–22		++(+)	>8		2000
21/Lu 3-032	C_2H_5	OH	D	base	127		+	>8		
22/Lu 3-017	=O		D	HCl	197	54	+	>8		>1000
23/Lu 3-027	C_2H (R = o-CH_3 C_6H_4-)	$C\,H_5$	D	HCl	214–16	48	+	>8		
R = C_6H_5 r = r_2 = CH_3	basic Sidechain									
24/Lu 3-042	-C-C-N<		D	HCl	204-06	40	++(+)	~1		700
25/Lu 3-054			M	oxalat	208–10	71	++	4		140
26/Lu 3-048	-C-C(-C)-C-N<		D	HCl	177–79	41	+	2	>64	
27/Lu 3-077	-C-C-C-N(H)-C-C			base	bp 1 mm 160–165		+	>8		>1000
28/Lu 3-043	-C-C-C-N(ring)N-C-C-OH			2HCl	230–35	68	0	>8		

D = Dimethylamino M = Monomethylamino * ED35 ** Never exceeds 50 %

Table 2

II INDANES (structure: indane ring with r_1, r_2, R and $(CH_2)_n-N<$)

1 Serial no. / code no.	R	2 r_1	r_2	n	3 Amino-group	4 Salt	5 Melting point C	6 LD50 mg/kg iv	7 Reserpine Antagon. Prelim.	8 Ptosis ED50 mg/kg ip	9 Reversal mg/kg ip	10 NA Potent. ED50 mcg/kg i
29/Lu 3-040	C_6H_5	H	H	3	D	HCl	165–67		++(+)	5		~1000
30/Lu 3-046					M	HCl	177–80	60	+++	~2	> 64	300
31/Lu 3-058	C_6H_5	H	CH_3	3	D	HCl	161–63	67	++	~8		
32/Lu 3-057α					M	HCl	188–90	62	+++	~2		150
33/Lu 3-057β					M	HCl	165–67	69	++(+)	4		710
34/Lu 3-060α	C_6H_5	H	CH	2	D	HCl	218–20	44	++	< 8		800
35/Lu 3-060β					D	HCl	206–07	64	+	> 8		
36/Lu 3-061α					M	HCl	172–74	67	++	3		170
37/Lu 3-061β					M	HCl	126–28	60	++	~4		680
38/Lu 3-047	C_6H	CH_3	CH_3	3	D	HCl	205–06	59	+++	3–4		2300
39/Lu 3-049					M	HCl	150–55	49	+++	0,9		110
40/Lu 3-067	m–CF_3–C_6H_4	H	H	3	D	base	bp 0,5 mm 125–30		+	> 8		

III INDENES

$(CH_2)_n-N<$

C_6H_5

		r	n								
41/Lu 3-064		CH_3	3	D	HCl	184–86	49	+	~6		
42/Lu 3-065				M	HCl	138–40	60	+	4		
43/Lu 3-083		H	2	D	HCl	199–201	45	+++	~.5		900
44/Lu 3-084				M	HCl	165–67	60	+++	~1		360
45/Lu 3-078		CH_3	2	D	HCl	206–08		+	>8		
46/Lu 3-079				M	HCl	130–32		+	<8		

Never exceeds 65%.

and a dose-effect curve was drawn on semi-logarithmic paper from which the ED50 was read. A few drugs were tested at a dose of 8 mg/kg to see whether maximal potentiation was still present or "reversal" had occurred.

Results

In tables 1 to 4 the compounds are identified in column 1 by code number or generic name. In order to facilitate reference to any one compound they have also been assigned a serial number (extreme left, column 1). As indicated under materials and methods, various bicyclic ring structures have been tested. Most of the structures (IV to XIV) listed in table 3 appeared of low or no activity and are thus only represented by a single prototype. Owing to their structural similarity with some of these groups methadone and its desmethyl-derivative were included (VI).

The most active compounds were found among the indanes (II, table 2), indenes (III table 2) and particularly the phthalanes (I table 1).

Among the phthalanes (table 1) by far the most active was Lu 3-010 (No 2) which was found about twice as active as the most potent of thymoleptics available hitherto protriptyline (No 74 table 4). Several structural characteristics, partly known from the studies of tricyclic compounds, appeared essential for this powerful reserpine antagonism and NA potentiation 1) phenyl substitution in position 1 2) substitution by two CH_3-groups in position 3 3) a three carbon sidechain, 4) a secondary amine group in the sidechain Compound Lu 3-071 (No. 10), which has no phenyl group, was of much lower activity than Lu 3-010, and so was Lu 3-053 (No 8) with a cyclohexyl ring in position 1 Substitution with Cl, CH_3 or CF_3 in the phenyl group likewise reduced activity It is apparently of some significance that just this presence of the phenyl group in position 1 establishes the closest resemblance of the bicyclic compounds to the "traditional" tricyclic thymoleptics. Reconstituting the C-C bridge between the phenyl groups, however, leads to complete loss of activity (Lu 3-056, No 59 table 3)

Substitution in position 3 appeared of great importance. One methyl group increased the activity over that of the unsubstituted compound, and two methyl groups increased activity even more, whereas C_2H_5 substitution decreased activity Similarly in the indanes two methyl groups increased the activity In the indenes, however CH_3 substitution appeared to have the opposite effect (compounds 45-46 vs. 43-44).

Including the previously known compounds in table 4 this investigation covered 24 pairs of dimethyl and monomethyl-amino derivatives. The general rule that monomethyl amines are considerably more potent

Table 3

OTHER RING STRUCTURES (Groups IV XIV)

1	2	3	4	5	6	7	8	9	10
						Reserpine Antagon.			
Serial / code no. / no.	Group'	Amino-group	Salt	Melting point °C	LD50 mg/kg iv	Pralim.	Ptosis ED50 mg/kg ip	Reversal mg/kg ip	NA Potent. ED50 mcg/kg iv
47/Lu 3-026	IV	D	HCl	178-80	37	++	~2		2200
48/Lu 3-036		M	HCl	199-201	61	+	~8		
49/Lu 3-023	V	D	HCl	207-09	71	+	>8		>4000
50/dl-Methadone	VI	D	HCl	235	32	(+)	>8		~1000''
51/Lu 3-062		M	HCl	170-75		(+)	~8		>1000
52/Lu 3-045	VII	D	2HCl	225	64	(+)	>8		
53/Lu 3-025	VIII	D	base	bp 0,5 mm 146	49	+	>8		
54/Lu 3-024	IX	D	2HCl	185	49	+	>8		
55/Lu 3-041	X	D	HCl	225-28		0	>8		
56/Lu 3-022	XI	D	HCl	215-17	61	+	>8		
57/Lu 3-039	XII	D	HCl	127 29		(+)	>8		
58/Lu 3-066	XIII	D	base	bp 0,5 mm 140-45		+	>8		
59/Lu 3-056	XIV	D	base	bp 0,1 mm 150-55	48	(+)	>8		

' See figure 1

'' Never exceeds 50%.

Table 4

TRICYCLIC COMPOUNDS and other reference substances.

1	2		3	4	5	6	7	8	9	10
							Reserpine Antago			
Serial no / code no			Amino-group	Salt	Melting point C	LD50 mg/kg iv	Prelim.	Ptosis ED50 mg/kg ip	Reversal mg/kg ip	NA Potent. ED50 mcg/kg iv
60/Melitracen	$=CH-(CH_2)_2-N<$ (9,9-dimethyl, Z-substituted)	H	D	HCl	241–43	52	++(+)	4	> 64	2500
61/Litracen		H	M	HCl	199–201	38	+++	2	> 64	780
62/Lu 3-011α		CF_3	D	HCl	237–40	68	0	> 8		> 3000
63/Lu 3-011β		CF_3	D	HCl	215–16	80	++	> 8		4000
64/N 7053	$-CH(H)-(CH_2)_3-N<$ (9,9-dimethyl, Z-substituted)	H	D	HCl	214–17	48	+	< 8		4000
65/N 7062		H	M	HCl	182–86		+	< 8		
66/Lu 3-004		CF_3	D	HCl	164–66		(+)	> 8		
67/Amitriptyline	$=CH-(CH_2)_2-N<$		D	HCl	197–99	38	+	14	32	~4000
68/Nortriptyline			M	HCl	217–18	39	+	6	32	2100

69/Lu 3–070	(tricyclic ring system)–H, –$(CH_2)_3$–N<	D	HCl	187	49	++(+)	1	64	~1000
70/Lu 3–063		M	HCl	173–75	67	+++	0,5	2	80
71/N 755	(tricyclic ring system)=CH–$(CH_2)_2$–N<	D	HCl	216–18		+	~4	8	>4000
72/N 7068		M	HCl	179–81	41	+++	~2	32	500
73/Lu 3–068	(tricyclic ring system)–H, –$(CH_2)_3$–N<	D	HCl	183–86	48	+++	0,5		2500
74/Protriptyline		M	HCl	166–67	49	+++	0,7	64	25
75/Imipramine	(tricyclic ring system)N–$(CH_2)_3$–N<	D	HCl	174–75	40	+	3	64	350
76/Desipramine		M	HCl	706–08	49	++(+)	1	32	70
77/dl-Amphetamine			H_2SO_4	>300	82	+++	2,5	–	7000
78/Cocaine			HCl	195	38	+	>8		260

than the dimethyl derivatives was confirmed One exception was found in the NA potentiating effect of the pair Lu 3–028 Lu 3–071 (Nos. 9 and 10), which are phthalanes with no phenyl group in position 1

Compounds containing two different substituents in position 3 afford the theoretical possibility for the existence of diastereoisomerism. Some α and β isomers were easily separated (compounds Nos. 32, 33 34 35, 36 and 37), but others have not been separated (compounds Nos. 13, 14 19 and 31) Differences in biological activity were sometimes seen between two diastereoisomers (32 vs. 33 and 36 vs. 37)

For rapid screening purposes the preliminary reserpine-antagonism test was found sufficiently reliable for differentiation between potent and weak compounds. However it must be realized that the employment of only two rather high dose levels does not do justice to compounds such as Lu 3–033 (19), amitriptyline (67), nortriptyline (68) and N 755 (71), in which the neuroleptic component reverses the antagonism at fairly low dosage In column 9 of the tables are indicated the dose levels at which reversal of reserpine antagonism was noted. It may be seen from table 4 that the dualistic action is a dominant feature of all known thymoleptics except melitracen (60) and litracen (61) Even the high thymoleptic activity of protriptyline is reversed at 64 mg/kg. In contrast, the most active compounds of the new series, particularly Lu 3–010 even at the high dose of 64 mg/kg, still showed 100/ antagonism to reserpine ptosis. A few attempts were also made in the pithed rat preparation to demonstrate this reversal phenomenon. An intravenous dose as high as 8 mg/kg Lu 3–010 still showed maximal potentiation of NA-pressor response (110/), but with protriptyline the potentiation was reduced to 28/ and with desipramine to 19/ Amitriptyline was lethal to pithed rats at this dose level. In motility cages Lu 3–010 does reduce spontaneous motor activity of mice, but a dose as high as 28 mg/kg is required for 50/ reduction, compared to 11 mg/kg for protriptyline and 5 mg/kg for amitriptyline. Thus Lu 3–010 appears to approach the ideal of a pure thymoleptic substance devoid of neuroleptic properties.

Another feature of the most potent phthalanes is their almost complete lack of anticholinergic activity On the conventional isolated guinea-pig ileum preparation the anticholinergic activity was measured relative to that of atropine. Lu 3–010 was found 1100 times less active than atropine, Lu 3–002 700 × < A, and Lu 3–051 1300 × < A, Lu 3–083 300 × < A, Lu 3–084 1200 × < A. Amitriptyline, melitracen and protriptyline are strong anticholinergics (10 9 and 11 times less than atropine)

Cocaine (78) is known to have catecholamine-potentiating properties. This is shown by the NA potentiation in pithed rats, which is a peripheral effect. But the antagonism against reserpine was weak and did not with

8 mg/kg reach 30 / Among the phthalanes Lu 3–028 (9) is another example of a substance with quite marked NA-potentiating effect, but with fairly low reserpine antagonism.

Amphetamine (77), which has been included in table 4 was shown to have reasonably good Na potentiating and antireserpine effects. But, in contrast to all other substances included in this series, amphetamine in effective doses induced signs of psychomotor stimulation before reserpine injection. Thus a clear distinction between amphetamine-like activity and thymoleptic activity was established for the new compounds also Further it may be stated that Lu 3–010 is completely free of monoamine-oxidase inhibiting effect, as are the previously known thymoleptics.

No relationship appeared to exist between thymoleptic potency and acute toxicity Generally the new compounds appeared less toxic than the previously known thymoleptics.

Summary

A number of bicyclic propylamines and ethylamines (cf fig. 1) have been investigated for "thymoleptic" activity (reserpine antagonism and noradrenaline potentiation)

The most potent compounds were found in group I, the phthalanes (fig 1) The 1-phenyl-3,3-dimethyl substitution revealed potent substances, particularly the secondary amine Lu 3–010 (No 2, table 1) which was found twice as active as protriptyline. Further Lu 3–010 in contrast to protriptyline and other known thymoleptics did not show reversal of "thymoleptic effects" and had no anticholinergic activity Like other thymoleptics, it had no monoamine-oxidase inhibiting activity or any amphetamine-like central stimulating effect.

Information added in the proof Further studies have revealed that besides l-phenyl-phthalanes also derivatives with other electronegative, non cyclic groups in the l-position have proved very active.

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For rapid screening purposes the preliminary reserpine-antagonism test was found sufficiently reliable for differentiation between potent and weak compounds. However it must be realized that the employment of only two rather high dose levels does not do justice to compounds such as Lu 3–033 (19), amitriptyline (67), nortriptyline (68) and N 755 (71), in which the neuroleptic component reverses the antagonism at fairly low dosage. In column 9 of the tables are indicated the dose levels at which reversal of reserpine antagonism was noted It may be seen from table 4 that the dualistic action is a dominant feature of all known thymoleptics except melitracen (60) and litracen (61) Even the high thymoleptic activity of protriptyline is reversed at 64 mg/kg. In contrast, the most active compounds of the new series, particularly Lu 3–010 even at the high dose of 64 mg/kg, still showed 100/ antagonism to reserpine ptosis. A few attempts were also made in the pithed rat preparation to demonstrate this reversal phenomenon. An intravenous dose as high as 8 mg/kg Lu 3–010 still showed maximal potentiation of NA-pressor response (110/) but with protriptyline the potentiation was reduced to 28/ and with desipramine to 19/ Amitriptyline was lethal to pithed rats at this dose level In motility cages Lu 3–010 does reduce spontaneous motor activity of mice, but a dose as high as 28 mg/kg is required for 50/ reduction, compared to 11 mg/kg for protriptyline and 5 mg/kg for amitriptyline Thus Lu 3–010 appears to approach the ideal of a pure thymoleptic substance devoid of neuroleptic properties.

Another feature of the most potent phthalanes is their almost complete lack of anticholinergic activity On the conventional isolated guinea-pig ileum preparation the anticholinergic activity was measured relative to that of atropine Lu 3–010 was found 1100 times less active than atropine, Lu 3–002 700 × < A, and Lu 3–051 1300 × < A, Lu 3–083 300 × < A, Lu 3–084 1200 × < A. Amitriptyline, melitracen and protriptyline are strong anticholinergics (10, 9 and 11 times less than atropine).

Cocaine (78) is known to have catecholamine-potentiating properties. This is shown by the NA-potentiation in pithed rats, which is a peripheral effect. But the antagonism against reserpine was weak and did not with

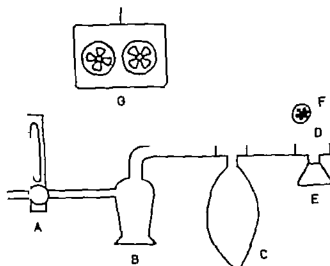


Fig. I Apparatus.
A Flowmeter B Nebulizer C Rubber bag. D Ruben's valve. E Face mask.
F Microphone G Tape recorder

Each subject had "training period" duri g which the concentration of citric acid producing 3–8 coughs was determined. This concentration varied from 5–7.5%. Lik BICKERMANN *et al.* we found the reaction to inhalation f citric acid to be strongest initially after 5–8 tests it dropped to an almost constant level.

Each test involved 3 inhalations f the fixed citric acid concentration, nebulized to 1 5 microns (air flow 8 l/min.). The response (number of coughs) were recorded on tape recorder. Each trial included 5 tests per subject the figures in the table represent the means of these 5 tests. The first part of the study was double-blind experiment with meprotixol tablets of 10 mg. Each subject received 3 tablets. As placebo we used glucose tablets f the same ppearance. The experiment extended over 2 days.

2. *Side effects*

T establish whether or not meprotixol in therapeutic doses produced side effects double-blind experiment was performed. The subjects were healthy persons, ged 24–40 years, 6 females and 4 males. They were told about the nature of the experiment beforehand. Doses of 20 mg Meprotixol were administered twice daily as placebo we used glucose tablets. Both were administered f periods of 10 days. When drugs were changed and at the completion of the experiment the subjects filled in questionnaire. This contained a list of the expected, nd blank space for possible unexpected, side effects. The questions were to be answered by none" questionable" or "unmistakable side effects.

Results

1 *Antitussive effect*

The result of the investigation is given in tables 1 and 2. The average reduction in the number of coughs was at a maximum after one hour and persisted for 3–4 hours.

Table 1

Reduction in number of coughs after 30 mg meprotixol by mouth.
The figures in brackets give the reductions as percentages.

	0	1	2	3	4 hours
R.E.	3.2	2.0 (38)	1.4 (56)	1.4 (56)	2.3 (28)
A.Ø	2.8	0.8 (71)	1.6 (43)	2.8 (0)	
J.M.	4.5	2.2 (50)	2.2 (50)	3.0 (32)	3.5 (18)
F.W.	3.4	1.0 (71)	1.6 (53)	2.6 (21)	
H.K.	4.4	2.2 (50)	2.5 (46)	3.4 (23)	
Mean in %		56	50	26	

Table 2

Variation in number of coughs with oral placebo.
The figures in brackets indicate the variations per cent.

	0	1	2	3	4 hours
R.E.	3.6	4.0 (+12)	3.6 (0)	3.0 (−18)	
A.Ø	2.0	3.3 (+50)	3.3 (+50)		
J.M.	3.8	3.5 (− 6)	3.8 (0)	3.4 (−11)	
F.W.	3.4	3.0 (−1)	2.8 (−18)	2.8 (−18)	
H.K.	5.4	4.6 (−15)	4.6 (−15)	4.6 (−15)	

Meprotixol was also administered intravenously to 7 subjects, in doses of from 20–35 mg. This part of the study was not performed as a double-blind experiment, because the investigator knew what was being administered. The subject was informed that he was receiving either active substance or saline. The results are shown in table 3.

Table 3

Percentage reduction in the number of coughs after meprotixol administered intravenously

Meprotixol		1		3	4	5 hours
35 mg	J.S.	31	16			
35 –	H.K.	74	69	42		
30 –	P.F.	47	20			
30	R.E.	66	45	39		
30 –	A.Ø	75	82	89	39	11
25 –	J.M.	63	53	42	29	
20 –	F.W.	77	41	25		
20 –	H.K.	0	0			

2. *Side effects*

The results are recorded in table 4. All the recorded side effects were described as doubtful.

It may be mentioned that in the study of the effect upon experimentally induced cough no side effects were observed when the drug was administered orally in doses of 30 mg. Two of the subjects experienced slight flushing after receiving 35 mg intravenously.

Discussion

1. *Antitussive effect*

Nearly all methods for the experimental investigation of the antitussive effect of drugs are beset by considerable sources of error (GRAVENSTEIN *et al* 1954). This also applies to our modification of Bickermann's method.

The factors producing coughs are: inflammatory conditions with oedema of the mucous membranes and accumulation of secretion, inhalation of smoke, dust, irritant vapours, etc. These irritants can be called physiological. After having tried inhaling citric acid, even at a low concentration, we are in no doubt that this irritant is at the upper limit of what may be called physiological. By using the concentration that gave a response of 3–8 coughs, we believe that we are at least as close as possible to a physiological irritant.

In order to obtain a constant irritation it is important that the concentration of citric acid in the air and the amount inhaled are constant. We kept the concentration constant by nebulizing the citric acid, throughout the experiment, at a flow of 8 l/min. However, the inhaled volume varies from one subject to another and also in the same subject in repeated experiments (BICKERMANN *et al* 1956).

This partly explains the small variation in the response to citric acid inhalation after the placebo. These variations were not marked in 4 of the subjects, (table 2). However, subject A.Ø showed, in one hour, an increase

Table 4

Side effects (all stated to be doubtful).

Symptom	Meprotixol	Placebo
Headache		1
Sleepiness		3
Tinnitus		1
Dryness of the mouth	1	

of 50% from the value at the time of administering the placebo Possibly the explanation of the low initial value is that, immediately before the test, the subject had administered 4 anaesthesias by vinydan ® (divinyl ether)/ether with an open mask.

When the subjects had received meprotixol, the irritability of the passages was clearly reduced. A few subjects stated that when they inhaled they felt the irritant effect of the citric acid far down into the chest, but did not feel any particular need to cough, although they could easily achieve one or more voluntary coughs Presumably the irritation grew more pronounced, because the volume of the citric acid/air mixture inhaled was greater than in the early part of the experiment.

2. *Side effects*

Meprotixol was administered in doses of 20 mg twice daily for 10 days to persons who showed no signs of disease. The single dose is at the upper limit of what would generally be needed to arrest cough on the other hand, the daily dose is at the lower limit of what would be needed to obtain a continuous antitussive effect.

In the experiment reported here the drug was administered to healthy people. The possibility cannot be ruled out, therefore, that it may accentuate existing symptoms when given to patients.

The subjects who had dryness of the mouth worked all day in a basement. The experiment was, by coincidence, carried out during a period of cold weather when the rooms were intensely heated and the air consequently unusually dry

Summary

Meprotixol has a good antitussive effect upon experimentally induced coughing in human subjects. After oral administration the maximum effect was attained in one hour and persisted for 3–4 hours.

No side effects were observed from doses of 20 mg twice daily for 10 days.

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Acta pharmacol et toxicol 1966, 24, 139–147

From The Department of Pharmacology University of Copenhagen
(Professor Knud O Møller M.D.)

Rate and Mechanism of the Renal Excretion of 14C Decamethonium by Rabbits

By

Chr Broen Christensen

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The renal elimination of decamethonium has been dealt with only in a few papers, and no reports seem to be available on investigations into its clearance rate and the mechanism of its renal excretion The compound being chemically a methonium salt, the excretion is likely to take place by glomerular filtration at a clearance rate near that of inulin

In 1950 ZAIMIS described a photometric method for determining methonium salts in urine. By this method she showed that the bulk of intravenously injected hexamethonium was excreted unchanged in the urine during the first 24 hours after its administration to cats, rabbits, or rats. Other workers have obtained similar results by the same procedure in experiments on rabbits and human subjects. (WIEN & MASON 1951 MILNE & OLEESKY 1951) ZAIMIS's method is, however not sufficiently sensitive for demonstrating decamethonium in urine after administering ordinary therapeutic doses. With a fairly rough biological method of assay based on the motor response in chickens after intravenous injection of decamethonium, a rapid renal elimination of decamethonium has been demonstrated after parenteral administration to human volunteers (PATON & ZAIMIS 1952)

The synthesis of isotope-labelled decamethonium has opened up the possibility of more exhaustive studies on its biological fate (WASER & LÜTHI 1957) The results of an investigation into the distribution of ^{14}C decamethonium in nephrectomised rabbits are given in a recent paper (BROEN CHRISTENSEN 1965). An account will be given below of an investigation into the renal excretion of ^{14}C-decamethonium after intravenous injection into rabbits. The object of this investigation was to study the mechanism of the renal excretion by simultaneous measurement of the

clearances of decamethonium and inulin To make sure that the ^{14}C activity measured in the clearance determinations represented unchanged decamethonium, the urine was also submitted to paper chromatography

Methods

Experimental

As experimental animals we used 15 male albino rabbits weighing from 1600 to 2600 g. The animals had been fasted for 16 hours before the experiments were begun, but had free access to water During the experiments the animals were strapped in supine position on an electrically heated operating table. Under halothane-N_2O-O_2 anaesthesia the animals had a cannula introduced into the trachea, a polythene catheter into the carotid and a short straight rubber catheter (Rüsch No. 8) into the bladder In all the experiments a single dose of 100 µg ^{14}C-decamethonium dibromide per kilogram were injected into the marginal ear vein in a carrier-free aqueous solution containing 100 µg/ml. The ^{14}C-decamethonium had been prepared by The Radiochemical Centre, Amersham, England, with a specific activity of 12.0 µc/mg.

Three long-term experiments, in which the urine was collected during the first 24 hours after the decamethonium injection, were carried out without tracheotomy and introduction of catheter into the carotid. The animals were kept in metabolism cages, where they had free access to beet-root and water At the conclusion of the experiment the bladder was emptied by suprapubic compression.

For *simultaneous measurements of the decamethonium and inulin clearances* a 1% inulin solution in 0.6% saline was infused at a constant rate (0.2 ml/min.) into the jugular vein. This was preceded by a priming dose of 10–20 ml of inulin solution. The animals were also given water through a stomach tube, maximum 50 ml/kg. Decamethonium was given intravenously in a single dose, as described above, about 30 minutes after the inulin infusion had begun. In each experiment urine was collected for clearance determinations during 60–90 and 90–120 minutes after the decamethonium injection. To ensure correct separation of consecutive urine portions, the bladder was washed with 10 ml of physiological saline at the beginning and end of each collecting period. Before and after the washing the bladder was emptied as completely as possible by suprapubic compression (Kruhøffer 1950). The urine flow can be estimated approximately by separate collection of urine samples and wash water Arterial blood samples for determining the plasma levels of decamethonium and inulin were withdrawn 57 72, 87 102, and 117 minutes after the decamethonium injection. The volume of blood withdrawn totalled about 12 ml per animal.

Administration of halothane ceased 5–10 minutes before the decamethonium injection.

Determination of decamethonium in plasma and urine

The plasma level of decamethonium was determined as described in a recent paper (Broen Christensen 1965). The ^{14}C-activity was measured by a liquid scintillation technique and converted into decamethonium concentration in plasma by comparison with standard plasma samples. The ^{14}C-activity in urine was likewise determined by liquid scintillation technique. An urine sample and the corresponding wash water were mixed and centrifuged, after which 200 µl of the supernatant were transferred direct to 10 ml of a scintillation medium (Bray 1960). The sample counting-rate was compared with that for standard urine samples prepared by adding known amounts of ^{14}C-decamethonium

to urine collected before the injection of decamethonium. As the samples may differ somewhat in colour and composition, the effectiveness of the measurement was controlled for each sample by a further measurement after addition of 25 µl of internal standard (toluene-14C, NEC 103 from The New England Nuclear Corp.). After correcting for quenching, the decamethonium content of the samples could be calculated on the basis of the counts for the standard samples. There was direct proportionality between the dded amount of decamethonium and the corrected counts. Counts of a blank collected within the preliminary period were used for background. Not less than 5000 counts were recorded per sample, and counts always amounted to more than five times the background value. The radioactivity was measured with a IDL scintillation counter type 6012 (Isotope Developments Ltd., England)

Determination of inulin in plasma and urine

For determination f inulin we employed BOJESEN's method (1952), which is based on formation of a blue colour with diphenylamine in an alcohol and sulphuric acid mixture t 100° We followed closely the procedure described by Bojesen, except that the volumes were reduced. After protein precipitation by the procedure of SOMOGYI (1930), 400 µl of plasma filtrate were pipetted ff and transferred to a glass ampoule. To this were dded 2 ml of diphenylamine reagent. The ampoule was sealed and then placed first in water bath at 100° for exactly 15 minutes and then in ice-water

The extinction was measured in the Beckman spectrophotometer DU at 650 mµ with bsolute alcohol in the blank cuvette. The colour intensity of glucose being only about one hundredth that of inulin, spontaneous fluctuations in the plasma glucose level will have no ppreciable effect on the result, provided the inulin concentration in the plasma is sufficiently high, i.e. exceeds 200–300 µg/ml. The extinction in plasma samples withdrawn before beginning the infusion was taken as the blank.

T measure the concentration of inulin in urine we used 400 µl of the mixture of urine and wash water which had been further diluted with distilled water in the ratio f 1 20. The sample was treated in the same manner as plasma filtrate. Duplicate determinations were carried out on both urine and plasma.

Calculation of clearances of decamethonium and inulin

The clearance was calculated as the U/P ratio, where U represents the average amount of the substance excreted with the urine per minut (µg/min.) nd P the plasma concentration of the substance (µg/ml). Allowance was made for the delay due to passage of the urine sample through the urinary tract and catheter ("delay-time"). Thus, in calculating the inulin clearance we used the plasma concentration of inulin three minutes before the middle of the rine-collecting period as representative of the experimental period. In calculating the decamethonium clearance we used the average decamethonium concentration in plasm during a period three minutes ahead of the urine-collecting period. The calculation was based on the individual curve for the decamethonium concentration in plasma determined by sampling blood every 15 minutes.

Chromatographic procedure

Chromatography of urine and the preparation to be injected was carried out by the ascending technique on Whatman paper no 1 As mobile phase we used two different systems A, ethanol, water ammonia (75 25 2) and B, n-butanol, ethanol, glacial acetic acid, water (8 2 1 3). The samples were applied with a Carlsberg pipette in narrow stripes (about 5 × 40 mm) at right angles to the flow direction in volume of 100 µl, and drying

was perf rmed with hot air An aqueous solution of non-labelled decamethonium (5–10 µg) was also applied as control. The chromatograms were developed at 20 for bout four hours and were then dried The ^{14}C-activity was scanned with an automatic chromatogram scanner (special design constructed by Philips Ltd Copenhagen f r the Isotope Laboratory, Rigshospitalet), stepwise f r each 1.5 mm. The distributio of activity was thereafter plotted, with counting rates as a function of the distance from the starting line. The R_f values were calculated as the ratio between the distance from the starting line to maximum activity d front respectively Th control spot was produced by spraying the chromatogram with Dragendorff reagent (potassium bismuth iodide), allowing decamethonium to be localised as a red streak on a yellow background (TOMPSETT 1964).

Results

The renal excretion of ^{14}C-decamethonium

The radioactivities measured in urine collected during 4 and 24 hours after intravenous injection of 100 µg ^{14}C-decamethonium per kilogram are shown in table 1 It is seen that about half of the injected dose had been excreted after 4 hours and about three quarters after 24 hours.

Fig. 1 illustrates the mean course of the plasma decamethonium concentration during the period of clearance determinations. Each point of the curve represents the mean of six experiments. In table 2 are given the results of the decamethonium and inulin clearance determinations carried out during two 30-minute periods on each animal. The weights of the animals averaged 1970 g (ranging from 1710 to 2270 g). The average *urine volume per minute was about 0 5 ml and about 0.4 ml,* respectively during the periods of 60–90 minutes and 90–120 minutes after the injection of decamethonium During the former period the decamethonium/inulin clearance ratio was 0.82 ± 0.11 during the latter period 0.84 ± 0.09 (mean $\pm$ standard deviation) In fig. 2 all the values for the decamethonium/inulin clearance ratio have been plotted against the corresponding values for the urinary flow

Table 1

Rat of renal excretion f ^{14}C-decamethonium after i. injection.

Period	N f experiments	Percentage excretion of injected dose
0–4 hours	6	51 ± 9 (M $\pm$ SD)
0–24 hours	3	75 (range 64–83)

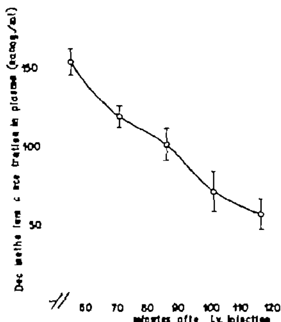


Fig. 1 The average time course of ^{14}C-decamethonium concentration in plasma after i.v. injection of 100 µg/kg in 6 rabbits.
The vertical bars indicate the standard deviation.

Abscissa: Time in minutes after injection.
Ordinate: Concentration of decamethonium in plasma (nanogram/ml).

Chromatographic identification

The results of the chromatographic analyses in a single experiment are shown in table 3. It is seen that the injected preparation was inseparable chromatographically from non-labelled decamethonium. Further both systems gave the same R_F value for the ^{14}C-labelled urine component as

Table 2

Renal clearance of decamethonium compared with simultaneous inulin clearance.

No. of experiments	30 min period no.	Average clearance (ml/min.)		Ratio for decamethonium/inulin-clearance (mean ± SD)
		decamethonium	inulin	
6	1	7.8	9.4	0.82 ± 0.11
		7.5	8.9	0.84 ± 0.09

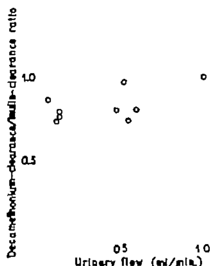


Fig. 2. Ratio for inulin/decamethonium clearance in relation to urine flow

Abscissa Urine flow (ml/min.).
Ordinate Ratio of inulin/decamethonium clearance.
Data from 12 experiments in 6 rabbits.

for authentic decamethonium, and no other activity peak was demonstrable in the chromatogram than that representing decamethonium. The same results were achieved in five other experiments, when the urine collected within two hours of decamethonium injection was submitted to chromatographic analysis.

Chromatography of urine samples later with system B (n-butanol, ethanol, glacial acetic acid 8 2 1 3) disclosed two activity peaks in

Table 3

Paper chromatographic identification f urinary ^{14}C-labeled compound after i injectio of ^{14}C-decamethonium.

	Solvent system*)	R_f value control	R_f value ^{14}C
Preparation injected	A	0.84	0.85
	B	0.48	0.48
Urine collected 0–120 min after injection	A	0.84	0.85
	B	0.48	0.49

*) Solvent systems were A, ethanol, water ammonia (75 25:2) and B, n-butanol, ethanol, glacial acetic acid, water (8 2 1 3)

several experiments, one representing decamethonium the other with a higher R_F value. The latter peak was not invariably present, and the suspected metabolite was of small quantity relative to the amount of activity administered.

Discussion

The rate of excretion noted for decamethonium is of the same order as that reported in the literature for hexamethonium. WIEN & MASON (1951), in experiments on rabbits, found up to 84% of intravenously injected hexamethonium to have been excreted in the urine within 24 hours.

We found the clearance of decamethonium to proceed at the same rate as that of inulin, which suggests that decamethonium is excreted by glomerular filtration. In this respect there is also agreement between our results with decamethonium and those achieved by other workers with hexamethonium. YOUNG DE WARDENER & MILES (1951) studied the mechanism of the renal excretion of hexamethonium by rabbits and found the hexamethonium/inulin clearance ratio to be nearly one. MCISAAC (1962) who measured the hexamethonium clearance in cats by an isotope technique, arrived at a hexamethonium/creatinine clearance ratio of 0.92.

It is worth noting that in most of the experiments the clearance values found for decamethonium were somewhat below those for inulin. A certain reabsorption (rediffusion) of decamethonium must therefore be assumed to take place in the tubules. We should expect the reabsorption to depend on the decamethonium concentration in the tubular urine and thus also on the urine volume. However, fig. 2, in which the decamethonium/inulin clearance ratio has been plotted against the corresponding urine volumes per minute, affords no basis for this assumption. The objection may be raised to our method of calculating the decamethonium clearance that a fixed value has been employed for the delay-time, irrespective of the urine volume discharged. We ought, perhaps, to have reckoned with a somewhat longer delay time for the urine sample from animals with low urine flows and accordingly with a lower decamethonium clearance than that calculated for animals discharging only small urine volumes. Variations in the delay time could have no appreciable influence on the inulin clearance determinations, because the plasma level of inulin was practically constant during the experiment.

Binding of decamethonium by plasma proteins might be a cause of a lower clearance of decamethonium than of inulin. Our study did not include investigations throwing light on this question, but some of the results achieved militate against the hypothesis of protein binding.

On the basis of our investigations into the course of decamethonium concentration in plasma after intravenous injection into nephrectomized rabbits, it was possible to calculate an initial volume for the distribution of decamethonium corresponding at any rate to the extracellular space (BROEN CHRISTENSEN & SCHOU 1963). The demonstration of a rapid passage through the capillary wall suggests that decamethonium is only present in a diffusible form in plasma.

Further the results of the clearance determinations at high urine flows showed that on several occasions the clearance of decamethonium was equal to that of inulin This also argues against the view of a partial binding of decamethonium by plasma proteins.

The chromatographic results prove that decamethonium was excreted unchanged in the urine during the period of clearance studies. The results suggested excretion of a metabolite later on but this question requires further investigation by means of a more sensitive chromatographic method or use of an isotope-labelled preparation with a high specific activity WASER has since 1957 been working with ^{14}C-decamethonium with a high specific activity has recently published the preliminary results of a metabolic study on cats (WASER 1963) In experiments involving only the first 40 minutes after administering decamethonium small amounts of a metabolite were found in extracts from liver tissue

McISAAC (1962) found no evidence of metabolism of ^{14}C hexamethonium in cats. Using paper chromatography he noticed that hexamethonium was excreted unchanged in the urine. Further no $^{14}CO_2$ was demonstrable in the expired air

In our studies on the fate of decamethonium in nephrectomized rabbits $^{14}CO_2$ was detectable in the expired air though in only small quantities. This finding supports the hypothesis of slow metabolism of decamethonium (BROEN CHRISTENSEN 1965)

Summary

Investigations into the renal excretion of ^{14}C-decamethonium after intravenous injection into rabbits showed about 75 / of the injected dose excreted in the urine during the course of 24 hours. Determination of the decamethonium clearance gave values of the same order as those for simultaneously measured inulin clearance.

Paper chromatography of urine samples showed decamethonium to be excreted unchanged within the first two hours after the injection.

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From the Department of Pharmacology of H. Lundbeck & Co. A/S, Copenhagen
(I. Møller Nielsen, D V M M.Sc.)
and the Poison Centre, Psychiatric Department, Bispebjerg Hospital, Copenhagen
(C. Clemmesen, M.D)

Effect of Certain Drugs upon Amitriptyline Induced Electrocardiographic Changes

By

M. Nymark and J Rasmussen

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In cases of human amitriptyline poisoning the cardiovascular consequences have been described as hypotension, tachycardia, electrocardiographic (ECG) changes and cardiac arrest (ANDERSEN & BRUUSGAARD 1964 SØRENSEN & ANDREASSEN 1964 MIKKELSEN & ROJEL 1965). The doses taken in suicide attempts have been in the range 10–50 mg/kg.

Even at the therapeutic level, i.e 1–2 mg/kg, amitriptyline has occasionally provoked ECG changes, particularly in certain older patients (SCHOU 1962 KRISTJANSEN & POULSEN 1963 OLSEN 1963).

Apart from tachycardia, these changes consist in varying degree of defects in conduction as manifested in atrioventricular block or bundle branch block or both. In massive poisoning these changes may give rise to bizarre ECG s, probably caused by shifting ventricular pacemakers polyectopia. Nevertheless, all the changes are fully reversible.

The same kind of ECG changes has been induced by experimental amitriptyline poisoning in guinea pigs (BOISSIER, SIMON & WITCHITZ 1965) and in dogs (SCHÄRER 1965) In the experiments reported here we produced these changes in rabbits with the object of using poisoning in this animal as a model for studying the effects of a series of drugs on the course of the poisoning, in order to gain some insight into the mechanism underlying the cardiac complications.

Material and Methods

New Zealand Whit rabbits of either sex, but predominantly males, weighing 2 to 4 kg were kept supine on a small animal operating table. To avoid muscle noise on the ECG tracings, the animals were next put under light anaesthesia with a gas mixture of 80 % N_2O

in O_2, 4 + 1 litres/minute. By means of commonly used gas flowmeters the mixture was administered with the Ayre technique. A mask consisting of a wide bore (40 mm) thin-walled rubber tubing, was employed securing a close connection to the muzzle and mounted on a stainless steel base with two fittings for the gas inlet and outlet tubes. The ECG standard leads were taken from intramuscularly implanted electrodes, and recordings were made on a direct writing two-channel oscillograph (Philips Oscilloscript, type Fe 250). Usually only readings of lead I and II were recorded, but a switch box allowed the subsequent lead III recording when required. Satisfactory ECG s were produced with a paper speed of 50 mm/second. The heart rate was counted from the ECG. The drugs were dissolved in or diluted with distilled water and injected intraperitoneally, intramuscularly or intravenously into a marginal ear vein in a volume of 1 ml/kg bodyweight. For infusion the drug was diluted in physiological saline, the drop speed being adjusted so as to give volumes of between 1 and 4 ml per minute. In some experiments a polyethylene cannula was inserted into the femoral artery under local anaesthesia, and the blood pressure was recorded on a smoked drum. After the experiment survival was observed for 24 hours.

The drugs employed were: amitriptyline hydrochloride, dichloroisoproterenol (DCI), propranolol (inderal ®), dihydroergotamine methanesulphonate, phentolamine (regitine ®), reserpine (serpasil ®), noradrenaline base, prostigmine (Roche), pyridostigmine (mestinon ®), acetylcholinechloride, atropine sulphate, ajmaline (gilurytmal ®), quinidine sulphate, procainamide hydrochloride (pronestyl ®), desacetyllanatoside C (cedilanid ®). The doses are all given in terms of the salt, except that of norepinephrine, which is given as base.

In evaluating the ECG a comparison was made with the trace obtained in the control period before dosing, further the following normal values given by SCHNEIDER (1933) were considered: PQ 0.05–0.074 msec and QRS 0.015–0.025 msec.

Results

Our first aim was to determine a dose that would constantly provoke the changes without killing the animal too soon, but on the contrary allowing a convenient duration for therapeutic attempts. It proved im

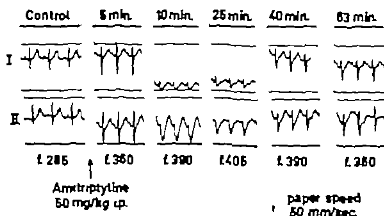


Fig. 1. ECG tracings from a rabbit, ♀ 3.3 kg, submitted to amitriptyline poisoning.

possible to satisfy these criteria by means of oral doses from 100 to 300 mg/kg. It was found that 50 mg/kg intraperitoneally provided the most reproducible results an example is shown in fig. 1 After this dose, only 5 of 49 rabbits did not respond with ECG changes. Fourteen rabbits died, seven apparently from the amitriptyline poisoning itself but the other seven as a result of the combined amitriptyline- antidote" poisoning. The time interval between the injection and the first changes varied from 3 to 10 minutes, and the duration was never less than one hour and was maximally recorded for 3½ hours Accordingly therapeutic attempts were limited to one hour after the amitriptyline injection. In three experiments performed without N_2O/O_2-anaesthesia changes of the same degree were

Table 1

Effect of different drugs on electrocardiographic changes in rabbits induced by amitriptyline 50 mg/kg i.p. See text for further explanation.

Drug tested	Dose mg/kg	Effect on heart rate	Effect on ECG changes	n	Survived 24 hours
Dihydroergotamine	0.1–0.5 i.m.	1 faster 3 slower	worse	4	0
Phentolamine	1.0 i.v	slowing	no	1	1
Dichloroisopropylnor adrenaline	2.5–5.0 i.m.	2 faster 2 slower	worse	4	2
Propranolol	0.5–5.0 i.m. i.v	slowing	no or worse	7	4
Propranolol pretreatment	0.5–1.0 i.v	slowing	prevention less 2 cases	7	6
Reserpine pretreatment.	0.1 1.0 i.v	slowing	no or slight prevention	3	2
Noradrenaline	0.001–0.003 i.v./min.	slowing	3 worse 1 better	4	0
Prostigmine	0.025–0.05 i.m.	slowing	no or better	4	2
Pyridostigmine	0.025–0.4 i.m.–i.v	slowing	normalizing	5	5
Acetylcholine	0.001 i.v./min.	slowing	no	1	1
Atropine pretreatment	2.0 i.	no	no	2	2
Ajmaline	1 0–10.0 i. –i.m.	slowing	worse	2	2
Quinidine	5.0 i.	slowing	slightly normalizing	2	1
Procainamide	10.0 i.	slowing	no or better	2	2
Lanatoside C	0.05 i.v i.m.	no	no	1	1

provoked, and in 4 experiments involving such anaesthesia, but without any amitriptyline medication, a normal constant ECG was recorded for 1–2½ hours.

In most experiments the possible antidote was administered when the ECG changes were manifest, but in some the possibility of preventing the changes by pretreatment with the "antidote" was investigated. The drugs used may be classified into those exerting an interference with adrenergic or cholinergic autonomic transmission and those with known anti-arrhythmic properties. The results are collected and summarized in table 1

A Interfering with adrenergic transmission

The α receptor blocking drug dihydroergotamine in a dose of 0 1 mg/kg i.m. reversed the amitriptyline-induced tachycardia to bradycardia. Increasing the dose to 0 2 mg/kg + 0.3 mg/kg 15 minutes apart provoked the same response, but 0.5 mg/kg in one dose increased the tachycardia. In all these tests a more pronounced bundle branch block resulted. All the animals died shortly after the end of the experiment.

The same bradycardic effect, but with no effect on the ECG was seen in one experiment with another α-receptor blocking drug, phentolamine, in a dose of 1 mg/kg i.v This animal survived.

Two β-receptor blocking agents, DCI and propranolol, were tested DCI in doses of 2.5 or 5 0 mg/kg i.m. slowed the heart rate by about 50 %, thus changing the tachycardia into bradycardia. The ECG changes were not affected on the contrary the QRS complexes tended to become even broader Two out of four rabbits died within 24 hours. The same effects were observed when propranolol was administered in doses of 5 mg/kg i.m., 0.5 mg/kg i.v + 2.5 mg/kg i.m 1 mg/kg i.v or 0.5 mg/kg i.v repeated 3 times 10–15 minutes apart. Two of these rabbits died during the experiment, presumably because of too high a dose of propranolol (5 mg/kg i.m. and 1 mg/kg i.v) On the other hand propranolol pretreatment prevented the occurrence of the ECG changes. In 4 experiments 1 mg/kg i.v given 5 minutes before amitriptyline was effective, and in two experiments only slight transient changes were seen. In yet another experiment 0.5 mg/kg i.v prevented the changes. Propranolol always induced bradycardia, which was not altered by the subsequent amitriptyline administration. Likewise the drop in blood pressure usually seen after amitriptyline was prevented. One of the pretreated animals died.

The significance of cardiac amines for the provocation of the ECG changes was examined by reserpine pretreatment. According to CARLSSON *et al* (1957) a dose of 0.1 mg/kg i.v after 16 hours had depleted the heart

of at least 90 / noradrenaline (NA) However this treatment failed to prevent the changes. Increasing the reserpine dose to 1 mg/kg, causing an almost complete depletion of NA from the heart, provided a slight protecting effect, sinus rhythm being preserved, but the QRS still broadened.

The transmitter itself in the form of NA infusions at a rate of 1 3 µg/kg/minute up to 20 minutes in one experiment showed a normalizing effect on ECG but in three cases the changes were increased. Some degree of bradycardia was induced. All four rabbits died.

B Interfering with cholinergic transmission

Two choline-esterase inhibiting drugs, prostigmine and pyridostigmine, were examined. Prostigmine in doses of 25 or 50 µg/kg i.m. produced some slowing of the heart and showed a slight normalizing effect on the ECG Two out of four animals died within 24 hours.

Another stigmine, pyridostigmine, proved to have a beneficial effect on the ECG changes In all but one case the trace normalized, sinus rhythm was restored, but the initial complex was still somewhat broadened. Further the amitriptyline induced tachycardia was much reduced. Pyridostigmine was given in divided doses ranging from 25 to 400 µg/kg i m in all All the animals survived.

Acetylcholine infusion at 1 µg/kg/minute produced some bradycardia, but was without any effect on the ECG changes. Atropine pretreatment, at 2 mg/kg i.v 10 minutes before amitriptyline, also had no effect on the course of the poisoning.

C Anti-arrhythmics

The rauwolfia alkaloid ajmaline which possesses antifibrillatory properties, *has recently been reported effective in different cases* of extrasystoles and paroxysmal tachycardia (LORDICK 1964) When ajmaline was administered in doses of 10 mg/kg i.m or 1 mg/kg repeatedly i.v bradycardia was produced and sometimes the P wave reappeared, but the QRS-complex was further broadened.

The two wellknown anti-arrhythmics, quinidine, 5 mg/kg i.v and procainamide, 10 mg/kg i.v., both provoked bradycardia and had a transient normalizing effect on the ECG Sinus rhythm was restored, but in one experiment the QRS complex broadened even more. With procainamide this effect was evident for about 5 minutes and could be produced again by an additional dose. In one experiment 4 doses of 10 mg/kg procainamide during one hour were given without deleterious effect.

The digitalis glucoside, lanatoside C, given in doses of 20 µg/kg i.m. + 30 µg/kg i v 45 minutes apart was without any detectable effects in one experiment.

Discussion

With amitriptyline poisoning, the ECG changes and especially the tachycardia could theoretically be produced by an inhibition of cardio-inhibitory vagal fibres or an enhancement of the sympathetic accelerant nerves or a combination of both. But a direct effect on the myocardium should also be considered.

The possibilities of marked local activity exist, as after amitriptyline administration relatively high amounts are bound in the heart as in other organs (HUCKER & PORTER 1961 CASSANO, SJÖSTRAND & HANSSON 1965)

The autonomic effects of amitriptyline are characterized by a strong anticholinergic atropine-like action and a relatively weak NA-potentiating effect (THEOBALD, BÜCH & KUNZ 1965 CAIRNCROSS 1965). The net result of these effects is a shift in autonomic balance towards adrenergic dominance. For the heart this means a positive chronotropic effect, i.e. tachycardia mainly due to the blockade of peripheral cardio-inhibitory vagal fibres but a contributing factor may be a potentiation of the effect of the sympathetic transmitter at the receptor site caused by amitriptyline's ability to inhibit the active amine pump function in the membrane of the synaptic terminals and thus the most important normal physiological inactivation of the transmitter (PLETSCHER 1964 CARLSSON & WALDECK 1965)

By itself such a shift to adrenergic dominance should not necessarily cause ECG changes On the other hand, it is well known that temporarily increased ventricular excitability as expressed by ectopic beats can easily be produced by injections of NA. When the normally occurring inactivation by uptake in terminals is then inhibited by amitriptyline, it seems not unlikely that such changes could be long-lasting and aggravated, particularly when at the same time the cholinergic cardiac inhibition is impaired. A contributing factor to this could be an uneconomic heart work caused by a decreased stroke volume and an increased load on the right heart.

On the other hand, one should consider also the dual action of amitriptyline on adrenergic mechanisms. Thus, at high dose levels the NA potentiating effect tends to reverse to an inhibiting effect. In such circumstances the receptor blocking properties dominate and overwhelm the opposite directed effects of the inhibited membrane pump (PETERSEN *et al* 1966). In this investigation involving high doses there was, however no evidence of reversal. This may be taken to indicate that this effect is limited to α-adrenotropic receptors and thus plays no part in the heart where NA combines with β-receptors (KOCH-WESER 1964)

When the sympathetic transmitter was almost completely depleted by reserpine pretreatment, the ECG changes were less pronounced. On the

other hand propranolol, which is a highly effective β-sympatholytic agent (SHANKS 1964 LEDSOME, LINDEN & NORMAN 1965), except for a bradycardiac effect was without beneficial effect on the ECG changes first established. However when propranolol was given before amitriptyline, the changes were prevented The discrepancy in these observations is not easy to explain, but differences in affinity for receptors in the heart, amitriptyline possessing the highest, might perhaps provide an explanation. In this connection it is worth noting that it has been demonstrated that amitriptyline, like catecholamines or sympathetic stimulation, activates the myocardial glycogen phosphorylase, which catalyzes the breakdown of glycogen to glucose 1-phosphate. Amitriptyline also potentiates the activating effect of isoprenaline on the heart. This has lead to the suggestion that amitriptyline brings about these effects by activating the myocardial β-adrenotropic receptors (SATCHELL, CAIRNCROSS & FREEMAN 1964) If it is correct that amitriptyline and propranolol compete for the same receptors, it seems that, once one of these drugs has occupied the receptors, there is a mutual insurmountable antagonism, i.e. irreversible competitive antagonism (VAN ROSSUM & ARIËNS 1962)

The finding of SCHÄRER (1965) that pyridostigmine can affect amitriptyline-induced ECG changes in dogs was confirmed, as for rabbits in fact this drug was the most effective antidote in our investigation and more effective than prostigmine. SCHÄRER suggested that the difference in effectiveness of the two stigmines was related to their different action on intestinal smooth muscle, thereby affecting the absorption of amitriptyline to a different degree. When i p injections were used, this mechanism is eliminated, and the difference in action therefore seems also to be related to a difference in the ability to inhibit cardiac cholinesterases. Pyridostigmine has been described as an almost specific antidote against human amitriptyline poisoning (MOESCHLIN 1965).

The normalizing effect of quinidine and especially of procainamide is most likely due to the prolongation of the refractory period (MOLLER 1965) accordingly although an ECG with all components was rebuilt for a short period, the QRS complex became even broader The failure of lanatoside C to affect the changes by increasing the contractile force is in accordance with clinical findings (MIKKELSEN & ROTEL 1965 J RASMUSSEN, unpublished observation).

In all species hitherto examined, including man, the ECG changes are fully reversible, which may be consistent with the assumption of transient autonomic dysfunction, but is also in accordance with the finding that amitriptyline disappears from the heart more rapidly than from other organs. After administering C^{14}-amitriptyline, high concentrations were found in the myocardium 5 minutes later but one hour later the con-

centration was low In contrast, radioactivity "was still noticeable after 24 hours" in the lungs (CASSANO *et al.* 1965)

In conclusion, although a direct cardiotoxic effect of amitriptyline may be involved, the most likely mechanism in the production of the ECG changes seems to be a heavily disturbed autonomic balance with an adrenergic dominance.

Summary

Rabbits given 50 mg/kg amitriptyline intraperitoneally were used for studying the mechanisms underlying the electrocardiographic changes produced by amitriptyline seen in human cases of amitriptyline poisoning. The effect of some drugs interfering with autonomic function or drugs with anti-arrhythmic properties on the course of the poisoning was investigated, and the results are discussed in the light of present knowledge of autonomic regulation It was concluded that the most likely mechanism in the production of the electrocardiographic changes is a heavily disturbed autonomic balance with a shift towards adrenergic dominance.

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From the Department of Pharmacology Institute of Pharmacy
University of Oslo

The Inhibitory Action of Cardiac Glycosides on the Isolated Guinea Pig Ileum
Effects of Alterations in the Ionic Composition of the Medium

By

Gudrun Bieltvedt and Kjell Briseid

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In a series of publications GODFRAIND & GODFRAIND-DE BECKER have reported on the effects of cardiac glycosides on the isolated guinea pig ileum (1961a & b 1962a & b 1963a & b) Their investigations were primarily directed to the second and inhibitory phase of action that follows a stimulating one. The results given in their papers might suggest the possibility of using the smooth muscle inhibitory effect of cardiac glycosides as a basis for their quantitative evaluation. Some difficulties involved in such an application, however were obvious the inhibition was persistent for a long-acting substance like digitoxin The slopes of the antagonist inhibition curves were rather low and the necessary concentrations of the glycosides relatively high.

In our work we altered the ionic composition of the Tyrode solution to obtain higher glycoside sensitivity and steeper inhibition curves. Further a procedure, based on the use of a temporarily increased concentration of potassium, was developed for washing the muscle free from glycoside relatively quickly Digitoxin and strophanthin-G were used as test glycosides, and a procedure for their quantitative estimation is suggested.

Technique

A *Modified Tyrode solutions*

During the inhibition period f the experiments we used a Tyrode solution modified to contain 66% of the usual amount f potassium (A) and, during the washing period introduced to free the muscle from its glycoside content, Tyrode solution altered to contain an amount of potassium 33% higher than usual (B). In both Tyrode A and B the calcium concentration was lowered t half the usual amount (table 1).

Table 1

Tyrode solutions used

Tyrode	g substance per 100 ml						
	NaCl	$CaCl_2$	KCl	$MgCl_2$	NaH_2PO_4	$NaHCO_3$	glucose
Normal	0.80	0.02	0.02	0.01	0.005	0.1	0.1
Modified A	0.81	0.01	0.013	–	–	–	
Modified B	0.80	0.01	0.027	–	–	–	

B. *Guinea pig ileum and apparatus*

Pieces of terminal ileum from fasted and freshly killed guinea pigs weighing 170–270 g were suspended in modified Tyrode solution A maintained at 34° (±0.1°). The muscle was attached to an isotonic frontal writing lever yielding a magnification of about 5. The load was 0.7–0.9 g.

The experiments were carried out in an automatic assay apparatus from Castle Electronics Ltd., London. The bath volume was about 3 ml, and the fluid was changed by emptying and refilling. The period of contact of agonist solution was usually 25 to 35 seconds (range 20 to 45 seconds). The washing consisted in replacement of the agonist solution with wash fluid and an exchange of the wash fluid with fresh wash fluid, which was left in contact with the muscle for 30 to 45 seconds before being again replaced by agonist solution. The interval between doses was fixed at about 2 minutes. The bath was aerated.

C. *Agonist and antagonist solutions*

Histamine at concentrations of 4 to 48 ng/ml was used as agonist.

Digitoxin and strophanthin-G at concentrations of 36 to 98 ng/ml and 100 to 195 ng/ml, respectively were used as antagonists. Both agonist and antagonist solutions were made up with Tyrode solution A.

D. *Materials*

Digitoxin. Sandoz A.G. Basel, Switzerland. *Histamine*. Histamine di-HCl, Light & Co Ltd. Colnbrook, England. *Strophanthin-G*. g-Strophanthinum cryst. Ouabain. E. Merck A.G., Darmstadt, Germany.

E. *Procedure*

After being suspended in the bath, the ileum was left for 20 to 30 minutes to relax, and then stimulation was begun with a submaximal dose of histamine. When the muscle became stable, the linearity of the agonist curve was checked by also giving two lower histamine doses logarithmically spaced. The antagonist solution was then left in contact with the muscle for 20 minutes (the solution was renewed after 10 minutes because of the smallness of the bath) and washed away and stimulation with the submaximal histamine concentration continued. After 11 doses the contraction level had just straightened out, and a new antagonist dose was given as described above. The increase in antagonist concentration in the modified Tyrode solution A was 25 or 40 %, according to the sensitivity of the muscle. If it became necessary to obtain two inhibition effects within the range 25–85 %, a third antagonist dose was applied in the same way and finally the linearity of the agonist curve was checked by three logarithmically spaced histamine doses. Agonist concentrations 3–4 times higher than before the first antagonist contact were usually required.

The muscle was then washed free from glycoside by replacing the modified Tyrode A with Tyrod B for 45 minutes, retaining the 2-minute dose cycle. Wash fluid was substituted for gonist solution during this period. When the washing period was finished, Tyrode B was replaced by Tyrode A and the muscle stimulated with histamine until the contractions had fallen to the original level, corresponding to th riginal potassium content. The muscle was then ready for contact with a new antagonist.

F *Assay of digitoxin and of strophanthin-G*

A quantitative estimation of an unknown glycoside solution required two experimental days. On the first day log-antagonist effect curves were established for a standard solution and then fo test solution. Each of the curves was based on two antagonist concentrations, as described under *Procedure*. On the next day the experiment was repeated on a piece of ileum from another guinea pig, but this time the unknown solution was tested first. The final result was calculated as the mean value f the results of the two days. The glycoside concentration of the test solution was calculated on the basis of the log-concentrations f test and standard solutions corresponding to 50% inhibitions of the submaximal agonist doses. Each log glycoside concentration corresponding to a 50 inhibition (log I_{50}) was calculated from two glycoside concentrations, causing inh bition in the range stated (25–85%) as shown in the following example 63 ng/ml (log = 1.80) of digitoxin caused 35% inhibition and 79 ng/ml (log = 1.90) 56% inhibition.

$$\log I_{50} = 1.90 + \left[(1.90 + 1.80)\ \frac{56 - 50}{21}\right] = 1.871$$

If both glycosid concentrations caused inhibition effects on the same side of the 50% level, the result was obtained graphically by extrapolation, but this was only considered permissible if one of the effects was within the inhibition range 45–55%.

Comments on the Technique

A. Modified Tyrode solutions

Significance of a reduction in calcium ion concentration Tyrode solutions with half the usual amount of calcium were not introduced especially for the experiments described here, but have been used generally in our laboratory to eliminate spontaneous contractions of the guinea pig ileum, which were otherwise sometimes troublesome Such a reduction in concentration of calcium ions reduced by about half the sensitivity of the gut to histamine. If the concentration of potassium ions was subsequently lowered to that of the modified Tyrode A (66% of the usual), the sensitivity of the muscle was further reduced by a factor of about 2.

The significance of such alterations in the ionic composition of the medium for the inhibitory effect of digitoxin is shown in table 2. In experiment *Ia* a log inhibitor-effect curve was first established in normal Tyrode, and subsequently – when the glycoside had been washed away in modified Tyrode B – also in a Tyrode solution with half the normal concentration of calcium. The results given in table 2 for this experiment suggest a considerably increased effect of digitoxin in the low calcium

11*

Table 2

Inhibition by digitoxin of the isolated histamine-stimulated guinea pig ileum.
Significance of alterations in the calcium and potassium ion concentrations.

Experiments 1, 2, and 3. *Comparison of inhibition* in normal Tyrode solution and in a Tyrode solution modified to contain (1) half the usual concentration of calcium, (2) ¾ of the usual concentration of potassium and (3) half the usual concentration of calcium and ¾ of the usual concentration of potassium.
Each of the three comparisons was based on two separate experiments, one of which began with the normal Tyrode (a), the modified Tyrode solution being used first in the other experiment (b).
For further details see text.

Digitoxin	Experiment 1a		1b		2a		2b		3a		3b	
	1/1 K, 1/1 Ca++	1/1 K, 1/2 Ca++	1/1 K, 1/2 Ca++	1/1 K, 1/1 Ca++	1/1 K, 1/1 Ca++	3/4 K, 1/1 Ca++	3/4 K, 1/1 Ca++	1/1 K, 1/1 Ca++	1/1 K, 1/1 Ca++	3/4 K, 1/2 Ca++	3/4 K, 1/2 Ca++	1/1 K, 1/1 Ca++
ng/ml causing 50 % inhibition	285	186	186	209	200	69	89	234	372	62	69	195
Difference ng/ml	99		23		131		145		310		126	
Difference %	35		11		66		62		83		65	
Average difference %	23				64				74			

Tyrode, 186 ng/ml against 285 ng/ml being required As, however the effective concentrations of the cardiac glycosides were often somewhat lower after the period of washing with modified Tyrode B experiment *1a* might overestimate the significance of the change in calcium ion concentration This was in fact demonstrated in a reversed experiment, beginning with Tyrode solution having half the normal content of calcium and ending up with a normal Tyrode solution. In this experiment (*1b*) the digitoxin concentrations causing 50% inhibition were 209 ng/ml for the normal Tyrode and 186 ng/ml for the low-calcium Tyrode The combined results from the 2 experiments show that a 23% lower concentration of digitoxin was required in the low-calcium medium. The log glycoside inhibition curves were somewhat steeper in the low-calcium Tyrode than in normal Tyrode

Significance of a reduction in potassium ion concentration The effect of a reduction in potassium ion concentration on the inhibitory action of digitoxin was considerably more pronounced than that of a reduction in calcium ion concentration. In a double experiment (*2a*, *2b*) designed as described above for the two calcium ion concentrations, it could be demonstrated that a 64 % lowered digitoxin concentration was required for a 50% inhibition of the agonist when the potassium content of the Tyrode solution was reduced to 66 % of the usual (1 8 mM against 2 7 mM) At the same time it was noticed that the change in potassium ion concentration steepened the log glycoside-inhibition curves. A further reduction in potassium ion concentration to half the normal (1.35 mM) was impracticable, as the agonist effect gradually declined. GODFRAIND & GODFRAIND-DE BECKER (1963b) observed in similar experiments that a decrease in potassium ion concentration from 2.7 mM increased the antagonism, which became unsurmountable when no potassium ions were added.

Significance of a simultaneous reduction in calcium and potassium ion concentrations (Tyrode A) Table 2 also shows the results of an experiment (*3a*, *3b*) in which the calcium and the potassium ion concentrations were reduced to those of the modified Tyrode A. The inhibition in this medium was of the same order as that observed in the Tyrode with low potassium and normal calcium concentrations, which shows that the sensitivity of the muscle to digitoxin was predominantly determined by the potassium ions. Several experiments demonstrated that there was required in the modified Tyrode A (in most experiments 40–80 ng/ml) about one fifth the concentration of digitoxin of that in normal Tyrode (200–400 ng/ml) for the same inhibition. The concentrations of digitoxin used by GODFRAIND & GODFRAIND-DE BECKER (1963a) to reduce by half the effect of histamine was 500–600 ng/ml or roughly 10 times higher concentrations than those

necessary in the modified Tyrode solution A to reduce the histamine contractions to one third to one quarter It should be pointed out, however that the authors mentioned used a 5-minute muscle-digitoxin contact period instead of the 20-minute period employed by us.

The persistence of inhibition after the contact time also varied with the Tyrode solution used. Though the inhibition in *normal Tyrode* in some experiments had already reached its maximum after about 7 histamine-caused contractions, and then slowly decreased again, the inhibition in the modified Tyrode A arose more slowly but was highly persistent. In one experiment the inhibition level was unchanged after an observation time corresponding to 22 histamine-induced contractions.

Significance of an increase in potassium ion concentration (Tyrode B). When the potassium ion concentration was *increased by 33 % from the* normal to 3 6 mM, the muscle could be washed free from glycoside in a 40–45 minute period. GODFRAIND & GODFRAIND-DE BECKER (1963b) have also pointed out the reduced inhibition at concentrations of potassium chloride over about 2.7 mM. Fig. 1 illustrates an experiment in which the digitoxin effect was eliminated as mentioned above. It will be noticed that the histamine contractions were considerably enhanced in the high potassium Tyrode, but were again normalized in the course of 30–40 minutes (15–20 cycles) when the modified Tyrode A replaced Tyrode B

B Procedure

Linearity of the log glycoside-inhibition curves The suggested calculation of log inhibitor values corresponding to a 50 % inhibition from two antagonist concentrations only was based on the linearity of the inhibition curves. Fig. 2 shows two linear curves, one with digitoxin and one with strophanthin-G as inhibitor and each based on 3 glycoside concentrations.

Period of contact between antagonist and muscle GODFRAIND & GODFRAIND-DE BECKER (1963a) employed a 5-minute contact time between glycoside and muscle in most experiments, but showed that the degree of inhibition increased with the contact period up to about 45 minutes. Our proposal of a 20-minute period represents a compromise between the time causing the strongest possible inhibition and the time most suitable from a practical point of view When the contact time was increased from 20 to 30 minutes, the increase in inhibition was not considered sufficient to justify advocating the longer period. The average inhibition values from several experiments carried out with a digitoxin concentration of 63 ng/ml were 36 % 60 % and 84 % for contact periods of 10, 20 and 30 minutes, respectively The slopes of the inhibition curves were

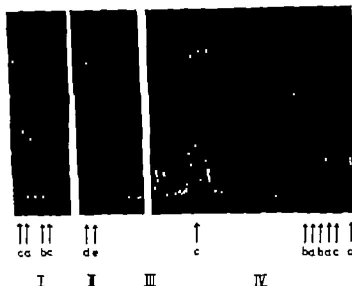


Fig. 1 Inhibitory effect of digitoxin on the isolated histamine-stimulated guinea pig ileum.
Removal of the glycoside by washing with Tyrode solution containing concentration of potassium chloride 33 % higher than usual.

I Histamine effect before digitoxin inhibition.
II. Histamine effect after two 20-minute contact periods with digitoxin, 70 ng/ml and 88 g/ml.
III. Washing period, 40 minutes with high potassium Tyrode solution.
IV Histamine effect after replacement of the high potassium Tyrode solution with the Tyrode solution originally used (66 % of the usual concentration of potassium chlorid).

Histamine concentration (a) 10 ng/ml
– – (b) 20 –
– ( ) 40 –
– (d) 60 –
– ( ) 120

The letters refer to the marked and the subsequent cycles.

nearly the same for the periods mentioned and so were the number of agonist contractions required for a stable inhibition level (about 11) and the persistence of inhibition.

Inhibition level When the antagonist solution had been removed, a stable inhibition level was ordinarily obtained after 8 to 11 cycles (16–22 minutes) A normal development of inhibition is evident in fig. 3, which shows the first stages of an experiment. Three log histamine doses demonstrate the linearity of the agonist curve after each of two successive periods of contact between the muscle and digitoxin (36 and 45 ng/ml), the contractions levelled out in the course of 9–10 and 10–11 cycles. The

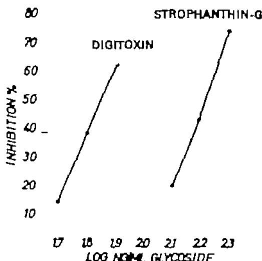


Fig. 2. Inhibition by cardiac glycosides f the isolated histamine-stim lated guinea pig ileum.

Linearity f log digitoxin d log strophanthin-G inhibition curves.

Both curves were based on 25 % increases in glycoside concentrations.

For details see text.

inhibitions recorded were 21 / and 69 / respectively It should be pointed out that in a few experiments 12–13 histamine-induced contractions were required to obtain an inhibition level after the first antagonist dose. The decreases in contraction heights observed being considered insignificant, a 11-cycle procedure was nevertheless adopted. The inhibition observed in modified Tyrode solution A was satisfactorily persistent. No increase in the inhibition level obtained had taken place after an observation time of 44 minutes.

Taking into consideration the persistence of inhibition, it was conceivable that the degree of inhibition obtained at a certain antagonist concentration might be due both to the effects of preceding antagonist doses and to the current concentration. To render any possible cumulation effect insignificant in determining the relative strength of a test and a standard preparation on one muscle, the same number of antagonist doses was used in both series. An experiment carried out, however with 3 digitoxin concentrations before the washing period, and afterwards with the highest digitoxin concentration alone indicated that the concentration itself was the determining factor and that the preceding antagonist doses did not contribute appreciably

Removal of glycoside in modified Tyrode solution B The contraction level obtained after a 40–45 minute washing period with modified Tyrode

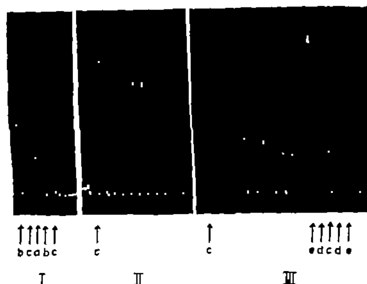


Fig. 3. Inhibitory effect of digitoxin on the isolated histamine stimulated guinea pig ileum.

Development of inhibition after two contact periods between the muscle and digitoxin.

Tyrode solution modified to contain 66 % of the usual concentration of potassium chloride.

I. Histamine effect before digitoxin inhibition.
II. Histamine effect after 20 minutes contact between the muscle and digitoxin, 36 ng/ml.
III. Histamine effect after an additional 20-minute contact period this time with 45 μg/ml of digitoxin.

Histamine concentration (a) 5 μg/ml
– – (b) 10 –
– (c) 20
– (d) 40
– – (e) 80

The letters refer to the marked and the subsequent cycles.

solution B was usually nearly the same as that observed before the first antagonist dose was added. In some experiments, however the final contraction level was considerably higher than the original level, and a prolongation of the washing period to 60 minutes did not reduce the contraction height. The increase in histamine effect might be an unspecific reaction of the muscle to the preceding inhibition, but it might also be due to an incomplete removal of the glycoside used. If the latter explanation is correct, the transitory potentiation of histamine often observed after the first antagonist dose probably demonstrates a stimulating glycoside effect caused by lower concentrations than those

resulting in inhibition. When the new contraction level did not exceed the original one by more than about 15–20 / the experiment was not significantly affected When the contraction level, however was more strongly potentiated, the experiment had to be rejected, as the slope of the inhibition curve subsequently obtained was then lower than usual

C. Assay of digitoxin and of strophanthin G

Inhibition effects of glycoside before and after the washing period. In a considerable number of the experiments the muscle was more inhibited after the washing period when the glycoside concentrations were retested. The fact was mentioned under "Comments on the Technique signif icance of a reduction in calcium ion concentration" and demonstrated in table 2. The increases in inhibition were often negligible and never exceeded about 10 / To eliminate any bias, however in the determination of an unknown glycoside solution, a two-day procedure was adopted based on an alternating use of standard and test solutions.

Assay No systematic series of glycoside assays was carried out to ascertain the precision of the procedure, as this was primarily intended for semi-quantitative comparisons of the effects of different cardiac glycosides. Table 3 however gives the result of an "assay" of digitoxin, in which the standard and test solutions were known and identical, and also of a similar "assay" of strophanthin-G Under these ideal conditions we

Table 3

Inhibition by cardiac glycosides of the isolated, histamine-stimulated guinea pig ileum.
Assays f digitoxin (I) and strophanthin-G (II).

Fo both glycosides the standard and "test" solutions were known and identical
F further details see text.

Assay	I				II			
Experiment	1		2		1		2	
Sequence f solutions	"Standard"	"Test"	"Test"	"Standard"	Standard"	"Test"	"Test"	"Standard"
g/ml causing 50 % inhibition	56	52	67	68	120	117	114	113
Relative activity "test /"standard"	108 %		101 %		103 %		104 %	
Average relat. activity "test"/"standard"	105 %				104 %			

found that with digitoxin the test solution had 105% of the activity of the standard solution, with strophanthin-G 104%

Comparison of the inhibitory effects of strophanthin-G and of digitoxin Under our experimental conditions the development and persistence of inhibition by strophanthin-G were fairly similar to that of digitoxin, and the slopes of the inhibition curves were the same for both glycosides. A determination of the relative strength of the glycosides was accordingly possible. The results of such an "assay" are given in table 4 which shows that strophanthin-G had about 52% of the ileum-inhibiting effect of digitoxin.

In experiment *2* of table 4 the inhibitory values observed for the highest glycoside concentrations were 92% and 94% for strophanthin-G and digitoxin, respectively which is beyond the inhibition range suggested as preferable (25–85%) Experience has however shown that, although inhibition values lower than about 25% were not reliable, as the curves tended to flatten out, higher values than about 85% did often fit well into the curves Table 4 further demonstrates that the relative activities observed for the glycosides in the two experiments were the same in spite of very different muscle sensitivities.

Summary

The sensitivity of the isolated guinea pig ileum to histamine stimulation decreased by a factor of 4 when the calcium and potassium ion concentra-

Table 4

Comparison of the inhibitory effects of digitoxin and strophanthin-G on the isolated histamine-stimulated guinea pig ileum.

For further details see text.

Experiment	1				2			
Glycoside	Digitoxin		Strophanthin-G		Strophanthin-G		Digitoxin	
Glycoside concentration ng/ml	70	98	156	195	100	125	50	63
Inhibition	29	72	45	76	40	92	31	94
ng/ml causing 50% inhibition	83		161		105		54	
Relative activity strophanthin-G/digitoxin	52%				51%			
Average relative activity strophanthin-G/digitoxin	52%							

tions of the Tyrode solution were reduced respectively to 50 % and 66% of the usual At the same time the sensitivity to digitoxin inhibition increased about 5 times, 40–80 ng/ml against 200–400 ng/ml being required for a 50 % reduction in the effect of a submaximal histamine dose. The alterations in ionic composition of the medium also steepened the log glycoside inhibition curve.

A method was developed for quantitative estimation of digitoxin and of strophanthin-G The glycoside concentration causing 50 % inhibition was determined as the average value from 2 separate experiments. The procedure adopted was based on 20-minute contact periods between the muscle and the glycoside and then 22 minute equilibration periods with histamine stimulation. The inhibition in the modified Tyrode solution used was persistent, but the glycosides could be removed from the muscle specimens during a 45 minute washing period if the potassium ion concentration was increased by 100 %

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From the Department of Pharmacology Institute f Pharmacy
University f Oslo

Determination of Plasma Kininogen, Plasma Kininase and Erythrocyte Kininase

By

Sidsel Flem Rinvik, Olav Kjell Dyrud and Kjell Briseid

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Determinations of the kininogen concentration and the kininase activity in plasma could give valuable information in connection with some pathological conditions. Such estimations would also be of interest for investigations into the effect of drugs on the kinin system of blood. Several authors have reported on kininogen determinations in plasma few however have given detailed information on the methods used (HAMBERG & ROCHA E SILVA 1957 DINIZ & CARVALHO 1963 AMUNDSEN, NUSTAD & WAALER 1963 FASCIOLO 1963 FASCIOLO & HALVORSEN 1964). Considerable deviations in the kininogen values observed might reflect fundamental differences in method. The elaboration of a selective method that determines different "kinin fractions" released by different enzymes would be too advanced a step to take at the present stage of knowledge about the substrate itself It has not yet been finally settled whether there exists more than one kininogen in the plasma of each species, nor is it certain which kinins are set free by the various liberating enzymes (BRISEID JENSEN, DYRUD & RINVIK 1966)

For the present it seems appropriate to employ a somewhat crude method that determines the total kininogen by maximal release of kinin. In this paper is described such a method based on a release of bradykinin by trypsin from acid-heated citrated plasma. Because of the known difference in rat uterus-stimulating activity of the kinins (kallidin/bradykinin = 0 6 on a weight basis), it is essential for the method of determination that trypsin should release only bradykinin from the final substrate.

Several authors have examined kininase activities in blood, urine and different types of cells. Their experiments, however did not aim at obtaining kininase activities suitable for quantitative comparison of results

from one time to another. No stable enzyme standard preparations were used, and the design of the experiments only made possible the establishment of fairly considerable differences in enzyme activities. In the work reported here we have tried to obtain reproducible determinations of plasma kininase and erythrocyte kininase by using a fixed amount of a substrate, bradykinin, in connection with a rigid time-inactivation relationship.

Results for plasma kininogen, plasma kininase, and erythrocyte kininase determinations in 11 healthy males are given.

Technique

A. Materials and Assays

1. *Substrate for determination of plasma kininogen.* A quantity of 9 ml blood was collected by venepuncture into a siliconized, graduated cylinder containing 1 ml of a 3.1 % sodium citrate dihydrate solution, transferred to a siliconized centrifuge tube and centrifuged at 1.3 × 10³g for 30 minutes at 10°. Of the citrated plasma (0.9 ml plasma) 1 ml was pipetted off and diluted with 9 ml of saline. 1 N hydrochloric acid was added to pH 2 (about 0.2 ml), and the sample was then heated for 30 minutes in a boiling waterbath. The sample was stored at −20°.

2. *Substrate for determination of plasma kininase.* Of the citrated plasma from the blood sample used for kininogen determination 3 ml were shaken for 30 minutes with 150 mg of silica powder. The silica was removed by centrifugation and decantation and the sample kept at −20°.

3. *Substrate for determination of erythrocyte kininase.* The erythrocyte fraction from the blood sample used for kininogen and kininase determinations was washed by means of a 4-times repeated suspending in an equal volume of 0.9 % sodium chloride solution and centrifugation for 15 minutes at 1.3 × 10³g. After the last centrifugation and decantation, two 1 ml samples were pipetted off and stored at −20°.

4. *Reagents. Bradykinin* (synthetic) in ampoules of 100 µg/ml, Sandoz, A.G. Basel, Switzerland. *Silicate powder* "Speed plus", Great Lakes, Carbon Corp., Los Angeles, U.S.A. *Trypsin*, 2 × cryst., washed free of salt, TRSF, Worthington Biochemical Corp., Freehold, New Jersey, U.S.A.

5. *Assays.* The kinin determinations were generally carried out on the isolated rat uterus as "bracketing assays" with a dose ratio of 3:2. Bradykinin was used as standard substance.

B. Methods

1. *Determination of plasma kininogen.* The boiled acidified substrate for determining plasma kininogen (about 10 ml with 0.9 ml plasma) was adjusted to pH 8.0 ± 0.2 with 0.1 N sodium hydroxide (about 2 ml) and incubated for 30 minutes at 37° with 0.45 mg of trypsin. It was then heated for 5 minutes in a boiling waterbath and diluted to 25.00 ml

with de Jalon solution. The stock solution was further diluted with de Jalon s solution and tested on the isolated rat uterus. When less than 3 μg of bradykinin was obtained per ml of plasma, the "bracketing" was repeated with trypsin (heat treatment as for the test solutions) added to the standard solutions. The results were expressed in terms of bradykinin.

2. *Determination of plasma kininase.* The method was in principle the same as previously described (DYRUD, RIMVIK & BRISEID JENSEN 1965). To various amounts of the silicate-treated, citrated plasma (for ex. 0.10 0.125, 0.15, 0.175 ml) were added 1.2 μg of bradykinin in 0.12 ml of 0.9 % sodium chloride solution and tris buffer (0.1 M, pH 7.3) to 1.00 ml. The mixture was incubated for exactly 8 minutes at 37° and the reaction stopped by adding 0.04 N hydrochloric acid t pH 2 (about 3 ml acid being needed) nd then heating for 10 minutes at 37° Just bef re assay 0.1 M sodium hydroxide was added to pH 8 0 ± 0.2, bout 1.5 ml being needed. The sample was diluted to 10.00 ml with de Jalon s solution and the stock solution further diluted for assay n the isolated rat uterus. A linear log-concentration response curve was drawn, and the plasma kininase activity was given as the mount of citrated plasma in ml per ml incubation mixture that inactivated 50 % of the substrate under the conditions described.

3. *Determination of erythrocyte kininase.* A 1 ml sample of washed cells was haemolysed by adding an equal volume of water and incubating for 5 minutes t 37° The rest f the determination procedure was as described for plasma kininase. The erythrocyte kininase activity was given as the amount in μl per ml incubation mixture of erythrocytes centrifuged for 15 minutes at 1 3 × 10³g in 0.9 % sodium chloride solution.

Comments on the Technique

A. Mat l ls

1 *Substrate for determining plasma kininogen.* The difference in rat uterus-stimulating activity f kallidin and bradykinin made it essential for the present method to be used with a substrate that would set free one kinin only on incubatio with the releasing enzyme. Bradykinin seemed easiest to obtain and was also preferable because f the higher activity on the uterus. BRISEID JENSEN, DYRUD & RIMVIK (1966) concluded from their experiments that the procedure of heating with acid suggested in the present paper resulted in a kininogen substrate that reacted readily to trypsin, releasing a kinin probably identical with bradykinin. The experiments indicated that no kininogen was lost thro gh the procedure.

Several previous uthors prepared their substrates for kininogen determination by heating with acid. VAN ARMAN (1955) added an equal volume of 0.3 % cetic acid to plasma and heated to 80–90° in waterbath HAMBERG & ROCHA E SILVA (1957) used the same method, and DINIZ & CARVALHO (1963) added 1.8 ml f 0.. % (/V) acetic acid to 0.2 ml of plasma and heated t 100° for 30 minutes in waterbath. The pH levels employed by the authors mentioned were higher than that suggested by us, but should, according to previous experiments (BRISEID JENSEN, DYRUD & RIMVIK 1966), suffice for converting the kininogen to release only bradykinin on trypsin incubation AMUNDSEN, NUSTAD & WAALER (1963), on the other hand, restricted their acid treatment to 10-minute incubation at pH 2 and 37° to destroy kininases, procedure that, according to our previous investigations, will only partly convert the kininogen. On subsequent incubation with trypsin or glandular kallikrein mixture of kallidin (or unknown kinins convertible to bradykinin through aminopeptidase) and bradykinin was released. FASCIOLO (1963) and FASCIOLO & HALVOR-

[illegible] (1964) used no acid treatment at all, but heated their plasma at 100° for 3 minutes to destroy inhibitors of trypsin and also peptidases present. If such a procedure inactivates all aminopeptidases in the plasma, a conversion of released kallidin to bradykinin cannot take place.

2. *Substrat for determination of plasma kininase.* ARMSTRONG, KEELE, JEPSON & STEWART (1954) and ARMSTRONG, JEPSON, KEELE & STEWART (1955 & 1957) demonstrated how kinins were released in plasma in contact with glass surfaces. The estimations of plasma kininase activities in our work being based on the inactivation of synthetic bradykinin added to the plasma specimens, any spontaneous release of kinins had to be avoided. MARGOLIS (1963) suggested that the activation of the Hageman factor by a foreign surface, as in silica powder would convert kallikreinogen to active kallikrein thus resulting in the release of kinins. He also pointed out how the treatment of plasma with sufficient silica powder would deplete the plasma of both the Hageman factor and other factors necessary for the release of kinins. AMUNDSEN, NUSTAD & WAALER (1963) found that a rapid passage of citrated plasma through a silica column altered the plasma specimens in such a way that they could not generate active kinin on storage in glass tubes at 37° or on dilution with saline. In our experiments we used a batch "stabilization" of the plasma specimens with silica powder. No spontaneous activity was observed after such treatment.

B. Methods

1. *Determination of plasma kininogen. Trypsin concentration.* The amount of trypsin suggested was adjusted so as to be sufficient for maximal release of bradykinin in substrates with a high content of kininogen. At the same time, however, the concentration of enzyme was kept as low as possible because of its uterus-stimulating effect. Table I shows that 0.5 mg/ml plasma was necessary for maximal release of bradykinin when the incubation was carried out at pH 8. 1 mg/ml, giving the same kinin value, but 0.25 mg trypsin/ml plasma did not suffice, and experiments with prolonged incubation period did not significantly improve the yield of kinin. An increase in the amount of plasma by 40% in experiments with 0.5 mg trypsin/ml resulted in an equivalent increase in bradykinin found, showing that this enzyme concentration gave sufficient margin of safety. Table I shows that incubations carried out with [illegible] mg and 4 mg trypsin per ml plasma resulted in somewhat higher bradykinin values. These results, however, probably reflect the ability of trypsin to stimulate rat uterus. The 5-minute heating of the test solution at 100° before assay, intended to denature the enzyme, was not sufficient to eliminate its effect on the uterus. Experiments with various amounts of trypsin showed that an enzyme concentration as low as 0.1 µg per ml de Jalon solution still slightly potentiated the response of the muscle to bradykinin. Attempts to eliminate the effect of trypsin on the uterus by increasing the 5-minute heating time to 15 to 30 minutes showed that 15-minute period was too short and that after 30 minutes the bradykinin amount found was somewhat reduced.

Usually the stimulatory action of trypsin on the uterus declined rapidly during the experiment. To make certain, however, of eliminating any bias in the results, trypsin was used also in the standard solutions when bradykinin values of 3 µg/ml plasma or less were obtained. DINIZ & CARVALHO (1963) generally used guinea pig ileum for their kinin assay but they also used the isolated rat uterus. It can be calculated from their results that the trypsin concentration in the final test solution was about 0.03 µg/ml for a human plasma that released an average amount of bradykinin per ml. A sensitivity of the uterus to bradykinin of 0.[illegible]–0.4 ng/ml is then assumed.

Table 1

Significance of trypsin concentration, incubation time and incubation pH for the determination of human plasma kininogen.

Substrate: Citrated plasma diluted 1 + 9 with saline, hydrochloric acid added to pH 2 and heated for 30 minutes at 100°
Trypsin concentration: 0.5 mg/ml fresh plasma.
Incubation pH: 8 0 ± 0.2.
Incubation time 30 minutes.
The incubates were heated for 5 minutes at 100° and assayed on the isolated rat uterus.
The trypsin concentrations are given as mg/ml fresh plasma and the kinin values obtained as µg bradykinin/ml fresh plasma

Trypsin mg	Bradykinin released µg	Incub. pH	Bradykinin released µg	Incub. time minutes	Bradykinin released µg
0.25	5 6	7.3	6.3	15	5 0
0.50	6.3	7.4	6.3	30	5.6
1.00	6.3	7.9	6.3	45	5 7
2.00	7 0	8.3	6.3	60	5 7
4.00	7 0	–	–	–	–

Incubation pH Table 1 demonstrates that the amount f bradykinin released was the same and maximal over the pH-range examined, 7.3 to 8.3 A pH of 8.0, which corresponded closely to the pH of de Jalon's solution, was accordingly chosen. DINIZ & CAR ALHO (1963) also found a pH optimum in the range 7.8 to 8.2, but in contrast to our results signif icantly lower yield of bradykinin at pH 7 4.

Incubation time. T ble 1 shows that an incubation time f 30 minutes was needed to give a maximal release f bradykinin. HAMBERG & ROCHA E SIL A (1957) showed that a maximum level f bradykinin was attained in 20 minutes DINIZ & CAR ALHO (1963) and FASCIOLO & HALVORSEN (1964) both used 30-minute incubation time. It is evident from table 1 that prolongation of the incubation time to 60 minutes caused no loss of kinin.

Reproducibility The precision of the kininogen determination method is shown in table 2. Eight different samples from the same plasma specimen were assayed at different times.

4. *Determination of plasma kininase and erythrocyt kininase.* The reproducibilities of the kininase determination methods are shown in table 2. For both a plasma kininase and an erythrocyte kininase preparation from the same subject 8 different samples were assayed at different times. Both kininases were determined without any previous purification procedure. Accordingly the amounts of "kininases determined do not reflect the basic amounts of the enzymes, but must be considered as the resultant effects of inactivating enzymes and possible accelerators and inhibitors. It has previously been shown that hydrocortisone in vitro at fairly high concentrations inhibited the inactivation of bradykinin (DYRUD, RENVIK & BREKKE JENSEN 1966).

Table 2

Determination of plasma kininogen, plasma kininase and erythrocyte kininase precisio of the methods.

I. Kininogen substrate.
II. Plasma kininase.
III. Erythrocyte kininase.
Of each preparation 8 separate samples were assayed at different times.
Fo details see text.

Sample	I Kinin released per ml fresh plasma as µg bradykinin	II ml plasma kininase preparatio per ml test solution causing 50 % inactivatio	III µl erythrocyte kininase preparation per ml test solution causing 50 % inactivation
1	6.3	0.19	13
2	6.3	0.20	8
3	5 7	0.23	12
4	6.3	0.20	13
5	6.3	0.20	13
6	6.0	0.25	15
7	6.1	0.25	15
8	6 0	0.19	14
Mean	6.13	0.214	12.9
s.e.m.	0.08 (1.3 %)	0.010 (4 7 %)	0.8 (6 2 %)

Results

Table 3 shows the results of determinations of plasma kininogen, plasma kininase and erythrocyte kininase in 11 healthy males within the age range 26 to 57 years.

Plasma kininogen

The average kininogen amount corresponded to a bradykinin release of 5.5 µg/ml fresh plasma, and the range was 4.2 to 6 7 µg/ml. From the values given by FASCIOLO & HALVORSEN (1964) it can be calculated that they obtained an average release of bradykinin in 3 experiments of 5.8 µg/ml plasma, which agrees well with our results. These authors used trypsin as releasing enzyme and assayed the kinin by its vasodilator action on the perfused dog leg preparation. The marked conformity with our results might seem somewhat surprising as the procedure used did not include any heating of the plasma at low pH and a mixture of kallidin and bradykinin should accordingly have been present (BRISEID JENSEN

Table 3

Determination of plasma kininogen, plasma kininase and erythrocyt kininase in 11 healthy males.

I Kininogen substrate Citrated plasma adjusted to pH 2 with hydrochloric acid and heated for 30 minutes at 100°

II Plasma kininase preparation Citrated plasma treated with silica powder for 30 minutes.

III. Erythrocyte kininase preparation Erythrocytes repeatedly washed with 0.9 % sodium chloride solution and centrifuged for 30 minutes at $1.3 \times 10^3 g$.

Test solutions for kininase experiments Kininase preparation + 1.2 μg bradykinin + 0.1 M tris buffer of pH 7.3 to 1 ml.

For further details see text.

	I	II	III
Subject number	Kinin released per ml fresh plasma as μg bradykinin	ml plasma kininase preparation per ml test solution causing 50 % inactivation	μl erythrocyte kininase preparation per ml test solution causing 50 % inactivation
1	5.0	0.14	8.5
2	5.0	0.18	5.0
3	4.2	0.18	6.0
4	6.1	0.10	7.5
5	4.6	0.13	5.0
6	5.8	0.17	5.0
7	6.7	0.17	10.5
8	5.7	0.19	12.5
9	4.8	0.20	5.5
10	5.6	0.20	4.5
11	6.7	0.17	5.5
Mean	5.47	0.166	6.86
s.e.m.	0.25 (4.6 %)	0.009 (5.4 %)	0.79 (11.5 %)

DYRUD & RINVIK 1966) If kallidin, as stated by WEBSTER & PIERCE (1963), is twice as active as bradykinin on the perfused dog leg preparation, the amount of kinin has been overestimated, bradykinin having been used as standard substance.

DINIZ & CARVALHO (1963) observed an average content of kininogen in human plasma corresponding to a release of 10.6 μg bradykinin (range 7.4–14.3 μg) per ml. The procedure described could be expected to give a kininogen that would yield only bradykinin on incubation with trypsin, and the authors assayed their samples against bradykinin on the isolated guinea pig ileum. No explanation can be given for their kininogen values being significantly higher than ours. It should be mentioned that heparin was used as anticoagulant, whereas citrate was used by us.

AMUNDSEN NUSTAD & WAALER (1963) on the other hand, found somewhat lower kinin values than those obtained in our work, 1 5 to 6 μg/ml fresh plasma. Two facts, however might explain their lower figures. First, they pretreated their plasma specimens at pH 2 and 37° a procedure that, according to our experience, will yield a mixture of kallidin (or another kinin transformable by aminopeptidase to bradykinin) and bradykinin. As they assayed their specimens on the isolated rat uterus, the presence of kallidin would cause an underestimation of the kinin amounts present, bradykinin having been used as standard substance. Secondly they regularly used saliva as source of kinin releasing activity which, like other glandular kallikreins, will probably cause only a partial exhaustion of the kininogen substrate.

Plasma kininase and erythrocyte kininase

No systematic quantitative studies seem to have been carried out on the kininase activities of human plasma or human erythrocytes. The methods of kininase determination used in the present work were based on a procedure developed for previous experiments on the *in vitro inhibition* of various kininases (DYRUD RINVIK & BRISEID JENSEN 1965)

Table 3 gives the results of determining plasma kininase and erythrocyte kininase in 11 healthy males. The average value of the plasma kininase concentrations causing a 50 % inactivation of the standard amount of bradykinin was 0 17 ml/ml, with a standard error of the mean of 0.009 (5 4 %) The average value of the precision experiments shown in table 2 was 0 21 ml/ml, with a standard error of 0 01 (4 7 %), which indicates that the variation between subjects in table 3 was mainly due to the error of the method.

The variation between individuals in the erythrocyte kininase determinations (table 3), on the other hand, was considerably larger than the method error the s.e.m. being 11.5 % against 6.2 %. Tables 2 and 3 show that both the erythrocyte kininase preparation and the plasma kininase preparation used for the precision tests were considerably less active than the corresponding enzyme preparations examined in order to determine individual variation. Since the material used for the precision experiments recorded in table 2 originated from one of the persons from whom results are included in table 3 the difference in activities might seem somewhat surprising. It should, however be pointed out that the precision material had been prepared by a slightly different procedure (DYRUD, RINVIK & BRISEID JENSEN 1965)

Summary

Methods have been described for determining plasma kininogen, plasma kininase and erythrocyte kininase in human blood.

The procedure developed for determining plasma kininogen was based on previous experiments on the release of kinins in human plasma substrate (BRISEID JENSEN, DYRUD & RINVIK 1966) It included a release by trypsin of kinin in citrated plasma heated at pH 2 for 30 minutes at 100° and a subsequent assay of the kinin on the isolated rat uterus with bradykinin as standard substance. The average amount of kinin released in 11 healthy males within the age range 26 to 57 years was 5.5 µg/ml fresh plasma with a s.e.m. of 0.25 (4 6 %).

No standard preparation was available for the kininase determinations, which were based on the amounts required for breaking down 50 % of a standard amount of bradykinin (1.2 µg/ml test solution) in a standard time (8 minutes) at pH 7 3 and 37° Results are given for the kininase activities in blood of the same 11 males who were used for the kininogen determinations, and the individual variation is indicated by the s.e.m. values (5 4 % for the plasma kininase and 11 5 % for the erythrocyte kininase determinations).

The reproducibilities of the methods were measured by determinations at different times of 8 separate samples of each preparation. The s.e.m. for the kininogen, the plasma kininase and the erythrocyte kininase preparations were respectively 1 3 % 4 7 % and 6.2 %

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From the Department of Pharmacology Institute of Pharmacy
University of Oslo

Determination of Plasma Kininogen, Plasma Kininase and Erythrocyte Kininase in Men with Rheumatoid Arthritis

By

Kjell Briseid, Olav Kjell Dyrud and Sidsel Flem Rinvik

(Received November 11 1965)

In a previous paper (BRISEID JENSEN, RINVIK & DYRUD 1965) values were given for the urinary excretion of kinins in males suffering from rheumatoid arthritis The mean daily excretion by 11 patients was significantly lower than that previously found in 12 healthy males (BRISEID JENSEN, VENNERÖD & RINVIK 1965)

In the work reported here methods elaborated for the determination of plasma kininogen, plasma kininase and erythrocyte kininase in human blood (BRISEID JENSEN DYRUD & RINVIK 1966 RINVIK, DYRUD & BRISEID 1966) were used to determine these factors in blood from 12 males with rheumatoid arthritis, patients at a hospital for rheumatic diseases (Oslo Sanitetsforenings Revmatismesykehus, Head Erik Kåss, M.D) The average values obtained were compared with the values previously determined in 11 healthy males (RINVIK, DYRUD & BRISEID 1966)

Technique

The collection of blood samples, the making of kininogen and kininase preparation and the assay of the active principles were as previously described (RINVIK, DYRUD & BRISEID 1966).

Results

The plasma kininogen, plasma kininase and erythrocyte kininase were determined for 12 males within the age range 38 to 74 years

Plasma kininogen. The amounts of kinin released were calculated as μg bradykinin, and the results are given in table 1 as μg kinin per ml fresh

Table 1

Determination of plasma kininogen plasma kininase and erythrocyte kininase in males with rheumatoid arthritis.

I *Kininogen substrate* Kinin released per ml fresh plasma calculated as µg bradykinin.
II. *Plasma kininase preparation.* ml kininase preparation per ml test solution causing 50% inactivation of a standard amount f bradykinin.
III. *Erythrocyt kininase preparation.* µl kininase preparation per ml test solution causing 50 % inactivatio of a standard amount of bradykinin.

Subject number	Treatment	I	II	III
1	Corticosteroid Salicylate	5.2	0.25	6 0
2	Corticosteroid Gold	4.6	0.17	4.5
3	Hydroxychloroquine Phenylbutazone Salicylate	4.9	0.30	9 0
4	Chloroquine Corticoster id Salicylate	4.5	0.30	9.5
5	Gold Salicylate	5 6	0.18	8.0
6	Salicylate	6.7	0.18	7.8
7	Gold Salicylate	6.2	0.18	7.5
8	Gold Hydroxychl roquine Salicylate	6.2	0.32	6 5
9	Gold Salicylate	6 2	0.27	6 0
10	Phenylbutazone	4.2	0.25	6 0
11	Indomethacin Salicylat	6.0	0.23	6.0
12	Salicylate	6 4	0.25	8 0
Means s.e.m.		5.56 0.25 (4.5 %)	0.240 0.015 (6.3 %)	7.08 0.38 (5 4 %)

plasma. The mean value was 5 6 µg/ml (s.e.m. 0.25) The corresponding value previously obtained in healthy males (RINVIK, DYRUD & BRISEID 1966) was 5 5 µg/ml (s.e.m. 0.25) The results thus showed that the kininogen levels in the plasmas of the rheumatoid patients, as measured by the maximum possible release of kinin, were normal

Plasma kininase and erythrocyte kininase Table 1 also shows the results of determinations of the kininase activities. The mean value for the erythrocyte kininase was 7 0 µl/ml (s.e.m. 0.38) The mean value previously obtained in healthy males (RINVIK, DYRUD & BRISEID 1966) was 6.9 µl/ml

(s.e.m. 0 79), which shows that the erythrocyte kininase level was normal in the patients with rheumatoid arthritis. The mean plasma kininase activity however was significantly lower in the arthritic patients. In the healthy males examined (RINVIK, DYRUD & BRISEID 1966) 0.17 ml plasma kininase preparation was required per ml test solution to cause 50% inactivation of the standard amount of bradykinin, whereas 0.24 ml/ml was the corresponding value in the patients. The standard errors of the means were 0.009 and 0 015 respectively This means that, on the average, concentrations more than 40% higher had to be used of the plasma kininase preparations from the subjects with rheumatoid arthritis than of the preparations from the healthy individuals. The observed difference was highly significant, a t test giving a P value of <0 001 No correlation was observed between the kind of drug used in medical treatment and the plasma kininase level

Discussion

When considering the apparently lowered activity of the plasma kininase in males with rheumatoid arthritis, it should be borne in mind that no purification of the kinin inactivating enzymes was carried out. The observed activities must accordingly be the resultant effect of the kininase and the accelerators, inhibitors and inactivators present. It has previously been shown that several substances used in the treatment of rheumatoid arthritis inhibit the kininase in *in vitro* experiments (DYRUD RINVIK & BRISEID JENSEN 1965) The lower level of the plasma kininase activity in the patients might thus be due to the therapy but it might also be due to the pathological condition itself If the latter should be so, the abnormality might be correlated with the pathophysiology of the disease or it might be an unspecific secondary phenomenon. The fact, however that we have observed not only a decreased excretion of kinins, but also a decreased level of plasma kininase in patients with rheumatoid arthritis, might point to the therapy as the cause of the low kininase level Assuming that the kinins in urine do originate from the blood, reflecting the plasma level, one would expect a low kininase activity to cause an increased and *not* a decreased kinin excretion. *It seems tempting to advance the hypo*thesis that the level of plasma kinins for some unknown reason is lower than normal in rheumatoid arthritis, and that kininase-inhibiting drugs used in the therapy may partly restore their normal level Other explanations, however are possible. For instance, if the amounts of kinins determined in urine do not reflect the plasma kinin level, the low kininase activity might still be related to the drugs used in the therapy of rheumatoid arthritis. In that event, however the effects of the drugs on the kininase might equally well be considered an unspecific side effect of substances that act through quite other mechanisms.

In the patients examined the level of kininogen was normal and so was the activity level of the erythrocyte kininase. It would be of interest to examine also the activity of the kinin-releasing mechanisms in subjects with rheumatoid arthritis compared to that in healthy individuals.

Summary

The concentrations of kininogen and the activities of plasma kininase and erythrocyte kininase were determined in blood from 12 males with rheumatoid arthritis.

The kininogen level of plasma was estimated by the release of kinin and calculated as μg bradykinin/ml plasma. The activities of the plasma kininase and the erythrocyte kininase were given by the volumes of the kininase preparations that caused a 50% inactivation of a standard *amount of bradykinin in a standard time under controlled experimental* conditions.

The average value of released bradykinin was 5.6 μg/ml (s.e.m. 0.25). The corresponding value previously obtained in 11 healthy males (RINVIK, DYRUD & BRISEID 1966) was 5.5 μg/ml (s.e.m. 0.25) which shows that the kininogen level in the plasma of the rheumatoid patients was normal.

The average value for the erythrocyte kininase was 7.0 μl/ml (s.e.m. 0.38), whereas the average value previously observed in 11 healthy males was 6.9 μl/ml (s.e.m. 0.79). The erythrocyte kininase level in the patients with rheumatoid arthritis was accordingly normal. The average plasma kininase activity on the other hand, was lower in the patients than in the normal subjects, 0.24 ml/ml test solution being required (s.e.m. 0.009) against 0.17 ml/ml (s.e.m. 0.015). The observed difference was highly significant ($P < 0.001$ by the t-test).

The apparent discrepancy between a previously observed low excretion of kinins in urine from males with rheumatoid arthritis, and this observation of a low plasma kininase activity in such patients, is discussed.

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From the central laboratory the FILADELFIA colony Dianalund, Denmark.
(Head dr phil. C. Munk Plum)

Identification of Meprobamate in Serum and Urine and its Quantitative Determination in Serum by Thin-Layer Chromatography

By

O Vendelin Olesen

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Numerous methods have been elaborated for the identification and quantitative determination of meprobamate in serum and urine since this drug first became available

After extraction by shaking, most of the quantitative procedures are based on colour reactions with hydroquinone in sulphuric acid (WALKENSTEIN *et al* 1958 ALLGREN *et al* 1959) or on the reaction between 4-dimethylaminobenzaldehyde and meprobamate in a non-aquoous medium (VIDIC 1959 HOFFMAN & LUDWIG 1959 MADSEN 1962) Methods that differ completely from these include, for example, splitting the meprobamate by means of sodium hydroxide and determining the ammonia released with Nessler's reagent (HARRIS & REIK 1958) or chlorinating the meprobamate and determining the bound Cl by means of potassium iodide possibly together with starch (ELLIS & HETZEL 1959)

The latter colour reaction has been used in various modifications for the identification of meprobamate by paper and thin-layer chromatography (VIDIC 1959 LINDFORS 1963 SUNSHINE 1963)

Among other colour reagents might be mentioned furfural and hydrochloric acid (MOSS & JACKSON 1961) and concentrated sulphuric acid, with heating (FIORI & MARIGO 1958).

The aim of our study was to elaborate a method that would be both quantitative and specific specific in order to demonstrate misuse of the drug, and quantitative to ascertain in serum cases of overdose or inability to excrete the drug.

Method

Serum

The serum is extracted with chloroform and the extract freed from fatty substances by transferring th meprobamate to a water phase, exactly as described in the chromatographic determination of phenytoin in serum (OLESEN 1965). The procedure for establishing standards and preparing the serum extract o the plates also follows the above method exactly except that the am ounts of the sample applied to the plate are 15 µl and 5 µl, respectively instead of 9 µl and 3 µl.

Adsorbent Kieselgel G (Merck), to which Leuchtpigment ZS Super (Riedel de Haen) has been added.

Length of run 6 cm.

Solvent Methan l, glacial acetic acid ether benzene, 1 9 30 60.

Chamber lined with filter paper

After drying, the plate is moistened thoroughly by spraying with a 2.5% solution of 4-dimethylaminobenzaldehyde in concentrated sulphuric acid and kept at 110 for 25 minutes. The meprobamate is thereby stained lilac-red. By comparing the size and intensity of the spots with those from standards (see fig. 1), it is possible to make a visual judgment of the meprobamate serum content alo g the lines already described (OLESEN 1965).

Urine

A sample of 5 ml urine is acidified with hydrochloric acid and shaken for 2 minutes with 25 ml of ether The ether is dried with sodium sulphate and evaporated in a w ter-bath, and the residue is dissolved in 100 µl ethanol. Amounts of 3 µl and 10 µl, or more if required, are applied to the plate.

Adsorbent substance As for serum analysis.

Length of run 10 cm.

Solvent chloroform, methanol, H_2O 75 25 1

The chamber is lined with filter paper and vessel filled with concentrated ammonia is placed in the bottom of the chamber

After drying, the colour is developed in the same way as for serum. Fig. 2 shows the developed meprobamate spots.

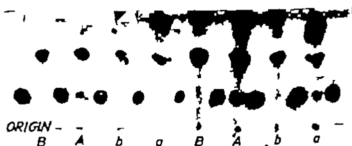


Fig. 1 Photograph illustrating meprobamate determination in serum.
A 3 ml serum and a 1 ml serum, respectively from a patient treated with 800 mg meprobamate daily are extracted, and f the extract $^{15}/_{40}$ and $^{5}/_{40}$, respectively are applied to the plate. B 3 ml serum and b 1 ml serum, respectively from a patient not treated with meprobamate, re given the same treatment.

The first line of spots bove the starting line are reddish-lilac meprobamate spots, and the spots above these are dark-coloured nd originate from normal serum constituents.

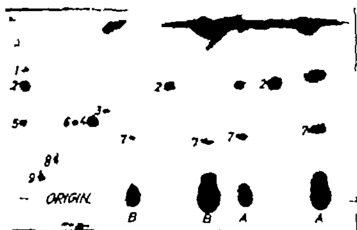


Fig. 2. Photograph illustrating identification of meprobamate in urine.
A: 5 ml urine from the same patient as in fig. 1 are extracted, and of this extract 1/100 and 1/1 are applied to the plate.
The same procedure is used to extract 5 ml urine from patient B in fig. 1.
Spot No. 1: Enhexymalum (light brown), 2: Meprobamate (reddish-lilac), 3: Methallymalum (brown), 4: 5-allyl-5-cyclopenten-(2)-ylbarbituric acid (bluish-black), 5: 5-(cyclohepten-(1)-yl)-5-ethylbarbituric acid (brown), 6: 5-(bicyclo(3,2,1)-octen-(2)-yl-(2))-5-ethylbarbituric acid (red), 7: unidentified dark spots, 8: Salicylamidum (bright red), 9: Salicylic acid (bright red).
The large dark spots on the starting line originate from normal urine constituents.

Recovery, accuracy and sensitivity

Recovery was determined by adding known amounts of meprobamate to a number of different sera, both from persons receiving no drug as well as from patients under treatment with a variety of drugs. Although recovery from the serum was only approximately 50 %, no effort was made to improve this, as the accuracy obtained, 1–2 μg/ml, may be regarded as sufficient for clinical use. Extraction of 3 ml serum makes it possible to demonstrate a content of approximately 0.5 μg/ml.

Experiments were also made in which four healthy experimental subjects received a single oral dose of meprobamate, 5 mg/kg, the serum concentrations being estimated 1, 2, 3, 4 and 24 hours after ingestion. These experiments have confirmed that the maximum concentrations in serum occur approximately two hours after ingestion (MADSEN 1962). Twenty-four hours after ingestion of the single dose, it was still possible to trace meprobamate in the serum of all 4 subjects.

Specificity

It is well-known that many drugs used in treating mental conditions develop a colour reaction on acid treatment. These drugs, however are

not extracted by ether from acid solution and so will not interfere in a urine investigation, where the chance of finding large amounts of these substances and their metabolites is optimal. Further their colour reactions on sulphuric acid treatment are instantaneous or develop in the course of a few minutes, whereas the strong colour that develops with meprobamate requires heating to 110° for about 25 minutes. A further characteristic feature is that, in contrast to most of these substances, meprobamate does not exhibit fluorescence in ultraviolet light.

It would lead too far to list those drugs not giving a colour reaction, but fig. 2 illustrates the separation of those drugs that I have found to give a reaction, although they show a variety of colours.

The solutions used to separate the spots differ The reason is that the methanol, glacial acetic acid, ether and benzene mixture used for the serum gives a better separation of meprobamate from other drugs that also react with 4-dimethylaminobenzaldehyde in concentrated sulphuric acid The Rf values are meprobamate 0.22, salicylamide 0.42, salicylic acid 0.55 and barbiturates approximately 0 48 This solvent, however is unsuitable for a crude urine extract, as a number of normal urine constituents will migrate with the solvent and be stained black on subsequent treatment by sulphuric acid and heat.

So far no substance has been found either in serum or urine that can give rise to confusion.

Discussion

Previously published methods for quantitative determinations of meprobamate with 4-dimethylaminobenzaldehyde not only needed special equipment, but were also critical with respect to the water content of the reagents, as a small amount of water displaces the reddish-violet colour developed in the blue direction (VIDIC 1959 MADSEN 1962) EIDEN & NAGAR (1964) have described the course of the reaction between meprobamate and 4-dimethylaminobenzaldehyde.

HYNIE *et al* (1965) identify meprobamate and metabolites in urine by means of the much employed unspecific chlorination method, as they did not find the reaction between meprobamate and 4-dimethylaminobenzaldehyde-hydrochloric acid sensitive enough. The method described in the present paper makes the identification of metabolites superfluous, as it is both sensitive and specific

In particular it is the barbiturates with cyclic non-aromatic substitutions that develop colours by the treatment described. In agreement with PAULUS (1963), I have found that cyclopental ① like dormovit ⑤ (5-

furfuryl-5-isopropylbarbituric acid) but in contrast to the other barbiturates, develops a colour with the reagent used in the cold

These substances can it is true, be removed from the chloroform or ether extract by shaking with a base, but this treatment has not been found necessary

Although the ingestion of a single therapeutic dose of meprobamate can be traced in serum 24 hours after ingestion, ordinary therapy has not shown any instances in which an accumulation of the drug has occurred Fig. 1 illustrates this for a patient who has been treated with 800 mg daily over a long period

Summary

A method is described for the quantitative determination of meprobamate in serum and the identification of the drug in serum and urine by means of thin-layer chromatography

The colour reaction employed is that between meprobamate and 4-dimethylaminobenzaldehyde in concentrated sulphuric acid.

The specificity must be regarded as being high and 0 5 μg/ml serum can be identified without difficulty when 3 ml of serum are used.

The accuracy achieved in quantitative determination in serum is 1–2 μg/ml.

The identification of the substance in urine is simple, as the procedure requires neither purification of the ether extract employed nor identification of metabolites.

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From the Department of Neurology (S. Refsum, M.D.), University Hospital, Rikshospitalet, Oslo, Norway and the Department of Physiology (H. Stormorken, M.D. V.M.D.), Veterinary College of Norway Oslo

Fate of Histamine and N Acetylhistamine Administered into the Human Gut

By

Ottar Sjaastad

(Received March 31 1966)

Free histamine and N-acetylhistamine may *a priori* gain access to the gastrointestinal lumen by any of several means, e.g. by release from the intact mucosa, by separation of mucosal cells, by ingestion, and by intraluminal production

Release of histamine into the gastrointestinal lumen is not likely to take place in healthy individuals (NORDLANDER 1957)

Histamine, usually in minute quantities, occurs in several foods (FELDBERG 1956 O SJAASTAD unpublished results) and N-acetylhistamine is present in e g. spinach (APPEL & WERLE 1959)

Bacterial production of histamine from L histidine is believed to take place intraluminally in healthy individuals (GALE 1946 IRVINE *et al* 1959a VANARSDEL & BEALL 1960 WATON 1963 BEALL 1965) There is, however a striking lack of positive evidence on this point.

N-acetylhistamine is the only histamine-metabolite known to arise intraluminally in man (URBACH 1949) After oral administration of ^{14}C histamine, an unidentified metabolite, differing from N acetylhistamine, was found in the upper one third of the small intestine of the rat (SCHAYER *et al* 1954)

Some details about the fate of orally administered histamine in man are known (ANREP *et al* 1944 URBACH 1949 ADAM 1950) This, however does not have any relevance to the fate of histamine possibly arising within the gastrointestinal lumen. In patients with dystrophia myotonica, normal

levels of histamine in gastric juice coexist with high faecal histamine-like activity and increased urinary output of N-acetylhistamine (O SJAASTAD, in press c) This observation demands an investigation of the fate of histamine and of N-acetylhistamine administered at various levels of the gastrointestinal canal, as reflected by the urinary and faecal excretion of free and conjugated histamine.

Material and Methods

Oral administration of histamine and N-acetylhistamine

Histamine diphosphate (Nutritional Biochemicals Corporation), 50–400 mg (18–144 mg histamine base)) was given by mouth to 5 healthy individuals (18 experiments). N-acetylhistamine (Calbiochem), equivalent to 0.23–190 mg histamine base), was given by mouth to 2 healthy individuals (11 experiments). The compound was dissolved in approximately 100 ml water and was administered just after breakfast.

Intestinal application of histamine and N-acetylhistamine

These substances were injected into various segments of the gut in patients undergoing gynaecological surgery for disorders such as fibromyomata, ovarian cysts or endometriosis. The patients had no complaints obviously related to the gastrointestinal tract.

These patients were given 0 1–0.2 g of phenylethylbarbituric acid (phenemalum N.F.N) on the night before operation. They were premedicated with 0.5–1.5 ml of a 5% solution of meperidine (pethidini hydrochloridum N.F N) and 0.6 mg of atropine sulphate.

Anaesthesia was induced by the intravenous administration of thiomebumal-natrium (pentothal-natrium ®, Abbott) and maintained with mixture of nitrous oxide and oxygen in a semi-open system. Suxamethoni iodid (curacit ® Nyco) was given as required. All patients were given 5% glucose and physiological saline intravenously during the surgical procedure, and most patients were given a 500 ml blood transfusion. Postoperatively, meperidine was administered for pain, usually in doses of 1–4 ml of a 5% solution.

Whereas 2 different doses of N-acetylhistamine were used, viz. 2.6 and 19 mg (a total of 5 experiments), standard dose of 72 mg histamine was used throughout the study (7 experiments) These doses were dissolved in 10 ml of sterile distilled ater The intestinal wall was perforated bliquely with thin needle to prevent leakage, and the doses were injected intraluminally The perforations in the colon were sutured.

Rectal instillation of histamine and N-acetylhistamine

Histamine, 72 144 mg, was administered rectally to 3 healthy individuals. The dose dissolved in about 5–10 ml of distilled water By means of glass funnel it was instilled approximately 10 cm above the anus, the test subject being in the recumbent position. The funnel was rinsed with couple of 1 2 ml portions of distilled water The instillations always performed just fter defecation. Experiments in which further bowel movement occurred before approximately 24 hours had elapsed were discontinued.

N-acetylhistamine, 0.46–190 mg, was administered to 3 healthy individuals (8 experiments), as described for histamine. In one experiment defecation occurred after 9 hours (compare table 6).

*) All doses of histamine and N-acetylhistamine given hereafter are calculated as histamine base.

Urinary xcretion of free and conjugated histamine

To the collection flasks 100 ml of 1.2 N hydrochloric acid was added, and this usually produced pH ≦ 1.5.

In the healthy individuals urine was collected from 0–6 hours and from 6–24 hours, or from 0–24 hours. On a few occasions the portion from 6–12 hours was collected separately. The surgical patients voided the urine just before anaesthesia was induced, and the injection was given 1 2 hours after this. Catheterization was not performed at the time of the injec tion. For these subjects the values for the 0–6 and 0–24 hour excretion will thus be slightly too high. Catheterizatio was performed in the surgical patients 6 hours after the injection.

Free and conj gated histamine were estimated as outlined by Duner & Pernow (1956). The procedure s described in detail elsewhere (O Sjaastad I press a).

The extraction procedure for surgical samples could not be begun until approximately 24 hours after the collection had been completed. If longer time had to elapse, the samples were frozen.

Control studies were carried out all healthy individuals and on some of the patients bout 2 weeks after surgery The interval between studies on a single test subject was at least 2 weeks. All test subjects were given a mixed diet.

Mean recovery in 25 experiments where 8–38 µg of N-acetylhistamine were added to urine was 74 ⁄ (range 94–59 %, S.D 8.6).

In the control series (O Sjaastad In press), the values for histamine in urine obtained (mean and range) were 12.6 and 31 µg/24 hours. The corresponding values for conjugated histamine were 30.0 and 1 49 µg/24 hours (unweighted values). The highest single observation for conjugated histamine was 130 µg/'4 hours.

The values for histamine and N-acetylhistamine, represent the mean of duplicate analyses, and the values are rounded off The figures given are not corrected for losses during the procedure. Calculations of the percentage of an administered dose excreted in urine or f eces, however, are invariably based on corrected values.

Faecal content of free and conjugated histamine

Faecal histamine-like activity (HA) was estimated as described in detail elsewhere (O Sjaastad, in press b). Mean recoveries of histamine nd N-acetylhistamine added to faeces were 60 % and 64 %, respectively. Only minute quantities of free and conjugated HA are found in the faeces from healthy individuals (HA < 0.17 µg/g wet weight) (O Sjaastad, in press b).

An antihistaminic gent (allergin ®) depressed the activity f the eluates and that of authentic histamine to the same degree.

Results

Oral administration of histamine

After oral administration of 72 mg histamine, the 24-hour urinary excretion of N-acetylhistamine increased by an average of 1138 µg. On average 32 ⁄ of the 24-hour excretion was found in the 0–6 hour samples and 45 ⁄ in the 6–12 hour samples When corrected for recovery the increase constitutes 2.1 ⁄ of the administered dose. The corresponding figures for doses of 144 and 36 mg were 3.3 ⁄ and 0.9 ⁄ respectively

There was a rather close correlation between oral dose and urinary

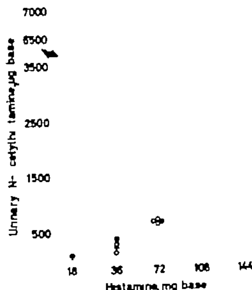


Fig. 1 Effect of oral administration of various amounts of histamine on urinary excretion of N-acetylhistamine. The values represent increments over basal excretion. Solid lines (—) indicate mean excretion for each dose.

excretion of conjugated histamine ($r = +0.70$, $p < 0.01$) (fig. 1). With doses of 108 mg histamine or less, the excretion of conjugated histamine from 24–28 hours was mostly within normal limits.

One test subject was given phthalylsulphathiazole 2 g q.i.d. for 5 days. The faeces were not cultured. Three hours after the last dose, 72 mg histamine was given orally. The ensuing urinary excretion of conjugated histamine amounted to 340 µg in 24 hours (164 µg in 6 hours). 1.4% of the administered dose was found in the faeces. It was only on this occasion that oral histamine caused severe abdominal cramp together with vertigo and faintness in the test subject.

The faecal excretions of HA in 3 experiments after 72 mg oral histamine are given in table 1. It was measured in 9 more experiments. The faecal excretion of HA varied between 0.5 and 11% of the oral dose and tended to increase relatively with increasing doses. A marked intra-individual variation in faecal excretion of HA was found with the same oral dose. If the first defecation occurred more than 4–6 hours after the ingestion of 72 mg or less of histamine, almost all HA excreted in the faeces was found in the first sample. Occasionally the amount of faecal HA after histamine administered orally proved remarkably stable.

With the regular technique for total faecal histamine estimation

Table 1

Urinary and faecal excretions of histamine after 72 mg base administered orally

Subject No.	Urinary conjugated histamine, µg base				HA
	0–6 hrs.	6–12 hrs	12–24 hrs	0–24 hrs	
I	200	350	130	680	6 hrs 14/2, 700 18 hrs 0.04/2.6
I	630	710	240	1,580	22 hrs 0.15/65
I				710	
II				3,000	10 hrs 0.1/4.8 33 hrs 0.02/1 4
II	470	610	500	1,530	
III				700	
IV				550	
V				750	

The figures represent histamine-like activity in 1 g of wet faeces and in the whole sample (µg).
1,000 µg excreted 0–6 hours.
Only total (free and conjugated) histamine-like activity measured.

(O SJAASTAD in press b), the presence of conjugated histamine could not be demonstrated with certainty

A small increment (level of significance 5–10 %, Student's t test) in urinary free histamine was found after oral ingestion of histamine (18 studies and 34 control studies in 5 individuals)

With doses of 36 mg histamine or more, various phenomena were experienced by some test subjects, e.g. nausea, belching, heartburn, borborygmia and diarrhoea. At times a feeling of warmness in the head associated with slight headache was noted

Histamine administered at various levels in the gastrointestinal tract

The urinary excretion of conjugated histamine invariably exceeded the control range after histamine administration at various sites in the intestinal tract (table 2) It should be noted, however that control studies were not always performed. The excretion after injection into the large intestine tended to be higher than the excretion after injection into the small intestine.

For urinary free histamine there was no clear trend. An increment was found in 1 of 4 subjects. Faeces were not examined.

No untoward effects of histamine injection were noted.

Table 2

Urinary excretion of conjugated histamine after administration of 72 mg histamine directly into the intestine.

Patient No.	Urinary conjugated histamine, µg base			Site of administration
	0–6 hrs	6–24 hrs	0–24 hrs	
1	470	810	1,300	By stomach tube, 2 3 hrs before operation
			1,300	50 cm below flica duodeno-jejunalis
3			660	Middle part of jejunum
4	9	420	430	2 m above Bauhin s valve
5	900	1,000	1,900	1 m above Bauhin s valve
6	450	5,000	5,500	Ascending colon
7			4,800	Ascending colon

Control studies were not undertaken.

Rectal instillation of histamine

The levels of urinary conjugated histamine were about the same after rectal instillation and after ingestion of histamine (tables 3 and 7). The faecal excretion of HA as a percentage of the administered dose ranged from 9 to 23 the mean being approximately 17 The highest value was found after the larger dose (144 mg histamine)

No clear trend for urinary free histamine was noted. No untoward effects were observed.

Table 3

Urinary and faecal excretion of histamine after rectal instillation of histamine.

Subject No.	Dose in mg	Urinary conjugated histamine, µg base			HA
		0–6 hrs	6–24 hrs	0–24 hrs	
I	72	125	550	680	22 hrs. 29
I	144	87	,300	2,400	27 hrs. 4
II	72			1,200	24 hrs. 7.5
VI	72	105	970	1 100	

HA means f ecal histamine-like activity mg base/g wet weight.

Table 4

Urinary excretio of conjugated histamine after N-acetylhistamine administered orally

Subject No.	Dose in mg	Urinary conjugated histamine µg base 0–6 hrs	6–24 hrs
I	190	69,300	19 600
I	19	11 100	2,100
I	19	4,900	1 100
II	19	10,000	3 100
I	2.6	1,280	210
I	2.6	1,500	320
II	2.6	1 180	450
I	1 7	960	160
I	0 87	330	80
II	0.87	300	240
I	0.23	150	22

Oral administration of N-acetylhistamine

The urinary excretion of conjugated histamine in these experiments is shown in table 4 About the same percentage of small as of large doses of N-acetylhistamine were excreted in the urine as conjugated histamine.

The faecal excretions of HA and conjugated histamine were examined in 5 experiments. No increment in excretion was detected, even after the largest dose of N-acetylhistamine, i.e. 190 mg base.

No significant increment in urinary free histamine took place after ingestion of N-acetylhistamine ($P > 0.05$). When considered alone, however the subject most studied (No 1 table 4) showed a significant increment in the urinary output of free histamine ($P \ngtr 0.05$).

Intestinal application of N-acetylhistamine

A significantly lower urinary excretion of conjugated histamine, as a percentage of the dose administered, was found after intestinal than after oral administration of N-acetylhistamine ($P < 0.001$ tables 4 5 & 7) All experiments of both kinds were used in these calculations, since there appeared to be no great difference in response to small and large doses.

The mean excretion of free histamine in these experiments was 9 6 µg/24 hours. Control studies without ingestion of N-acetylhistamine were not performed on these subjects. Faecal excretion of histamine was not evaluated.

Table 5

Urinary excretion of conjugated histamine after administration of N-acetylhistamine directly into the intestines.

Patient No	Dose in mg	Urinary conjugated histamine			Site of administration
		0–6 hrs	6–24 hrs	0–24 hrs	
8	2.6			760	m above Bauhin's valve
9	19	2,500	1 600	4,100	1 m above Bauhin's valve
10	19	5,000	900	5,900	1 m above Bauhin's valve
11	19			3,700	½ m above Bauhin's valve
12	19	3,200	1,500	4,700	Transverse colon

Rectal administration of N-acetylhistamine

After instillation of *small doses* of N-acetylhistamine, the increase in urinary output of conjugated histamine as a percentage of the administered dose was small. After the lowest dose in test subject No I (table 6), the excretion was of the same order of magnitude as the control excretion. The relatively high 0–6 hour excretion as compared with that from 6–24

Table 6

Urinary excretion of conjugated histamine after N-acetylhistamine administered rectally

Subject No	Dose in mg	Urinary conjugated histamine		
		0–6 hrs.	6–24 hrs	0–24 hrs
I	190	6,800	8,900	15,700
I	19	4 000	4,500	8,500
VI	19			2,100
II	3.5	190	390	530
I	2.3	170	57	230
II	0.93			25
II	0.46	54	33	87
I	0.46	61	[illegible]	86

Involuntary defecation after 9 hours. The faecal sample was not examined. Faeces after 23 hours contained 25 mg base total (free + conjugated) histamine-like activity of which 3.3 % was free histamine-like activity.
Approximately 56 % of the total histamine excreted in the faeces represented histamine-like activity

Table 7

Effect of administration of histamine diphosphat and N-acetylhistamine on urinary excretion of conjugated histamine.
(Condensed results from tables 1–6)

		Mode of administration	No. of exp.	Dose, mg base	Per centage excreted urine in 24 hours	Ratio 6/24 hours
Histamine diphosphate						
		Oral administration	18	144–18	2.1	0.32
		Oral administration (pretreatment with phthalylsulphathiazole)	1	72	0.6	0.48
	During surgery	Administration into stomach or small intestine	5	72	2.1	0.28
		Injection, large intestine	2	72	9.6	0.08
		Rectal instillation	4	144–72	1.9	0.11
N-acetylhistamine						
		Oral administration	11	190–0.23	77	0.79
	During surgery	Injection, small intestine	4	19–2.6	34	0.73
	During surgery	Injection, large intestine	1	19	34	0.71
		Rectal instillation**	2	19	38	0.47
		Rectal instillation	5	3.5–0.46	9	0.61

The figures represent averages of corrected values.
The dose of 190 mg is excluded from this table since defecation took place after 9 hours.

hours, however probably indicates that some of the N acetylhistamine was excreted as such in the urine in this experiment. HA or conjugated histamine could not be detected in the faeces.

With *higher doses* of N-acetylhistamine, a somewhat higher percentage of the administered dose could be recovered from the urine. In the experi-

ment with 190 mg N-acetylhistamine (table 6), defecation took place at 9 hours, and the faecal sample could unfortunately not be examined. Only 4 9 / of the dose was excreted in the urine within 6 hours.

With large doses of N-acetylhistamine, HA could be detected in the faeces (table 6) Of the 23-hour faecal sample from test subject No. I (table 6) 2 g were diluted with distilled water and centrifuged. A measured portion of the supernatant fluid was then subjected to descending paper chromatography (Whatman No 1) with N butanol/acetic acid/distilled water (4 1 1 v/v) as moving phase. The chromatogram was stained with diazotized sulphanilic acid. A clear spot was found with the same Rf value as that of N-acetylhistamine and a double-spot with the same Rf value as that of authentic histamine diphosphate.

No specific trend was noted for urinary free histamine. Thus in experiments with doses ≦ 19 mg, the output of urinary free histamine was lower than the mean control excretions for the individuals concerned.

When 190 mg were given rectally severe tenesmus was felt otherwise no unpropitious effects were noted with N-acetylhistamine, irrespective of route of administration.

Discussion

There is room for speculation as to how far the surgical procedure itself affected the results obtained in the female patients Stress has been claimed to facilitate intestinal absorption of histamine in guinea pigs (Selye 1938). Systemic effects were not noted in our patients receiving histamine In spite of possibly additional histamine of intestinal origin, urinary free histamine did not rise.

Further meperidine appears to be a potent histamine releasing agent (Zeppa *et al* 1961) The doses used for our patients were, however rather small compared with those used by Zeppa *et al* The normal excretion of free urinary histamine by the patient series tells against histamine release.

In general, anaesthetics do not seem to interfere with intestinal absorption (Wilson 1962). Finally intestinal motility was lively during and after the surgical procedure.

Conjugated histamine has previously been administered only to dogs. After oral administration, 50–60 / was excreted unchanged in the urine, whereas after subcutaneous injection it could be recovered, almost quantitatively from the urine (Anrep *et al* 1944) In our experiments, a somewhat larger proportion was excreted unchanged in the urine after ingestion whereas a smaller proportion was excreted after administration into the ileum. This may point to intraluminal catabolism of N-acetylhistamine.

Of small rectal doses of N-acetylhistamine, only a small fraction was recoverable from the urine, and none from the faeces With larger doses, the free fraction was detected in faecal samples. These findings indicate that intraluminal decay of N acetylhistamine takes place.

Histamine administered orally leads to a marked rise in urinary output of N-acetylhistamine (ANREP *et al* 1944 ADAM 1950 URBACH 1949 TABOR & MOSETTIG 1949), the N acetylhistamine, according to URBACH (1949), being formed in the intestinal tract.

ADAM (1950) found that between 0.17 and 1 / of orally administered histamine was excreted as conjugated histamine in the urine. In our experiments 2.1 / was excreted in the urine as conjugated histamine. The discrepancy may partly be explained by the fact that in our study *histamine was given just after a meal, since MELLANBY (1916) found that* absorption of histamine in the cat was delayed in the presence of food. In these circumstances, histamine may gain access to segments with an abundant bacterial flora and an ensuing greater degree of acetylation

Because of the insensitivity of the method, ADAM (1950) could not establish whether a significant rise in urinary free histamine occurred after ingestion of histamine. SCHAYER (1956) in a single experiment found no ^{14}C histamine in the urine after ^{14}C-histamine by mouth. In our study no significant increase in urinary free histamine was observed.

In dogs, LIVINGSTONE & CODE (1955) and IRVINE *et al* (1959b) found a temporary (4–5 hours), but clear increase in urinary free histamine after administration of histamine by mouth and after injection into the jejunum In man, such a small increment might easily be swamped in a 24-hour sample owing to the normal fluctuations in excretion. Their findings do not necessarily reflect the pattern in man MITCHELL & CODE (1954) in a small series followed the urinary excretion of free histamine in 2-hour periods after histamine by mouth. They found no rise, but the oral dose of histamine was rather small. Symptoms and signs possibly attributable to a systemic histamine effect were noted after oral histamine in our experiments. It is noteworthy however that these systemic effects provide no clear proof that histamine enters the general circulation unchanged. It is entirely possible that these effects are caused by histamine metabolites, of which several are biologically active (KAPELLER ADLER & IGOO 1957)

A conspicuous consequence of administering histamine to various segments of the gut was a pronounced rise in urinary conjugated histamine.

There is some evidence that urinary conjugated histamine has a dual origin, i.e. coming both from the intestinal lumen and from the tissues (URBACH 1949 KOBAYASHI 1957 DUNÉR & PERNOW 1960 O SJAASTAD, in press a).

Additional evidence for extraluminal conjugation is provided by our study the small intestine is as a rule practically devoid of resident bacteria (CREGAN & HAYWARD 1953) Moreover our patients fasted approximately 16 hours before surgery In one experiment X ray contrast (gastrographin ®) was injected into the upper jejunum together with 72 mg histamine. On this occasion 120 µg conjugated histamine was excreted during the 6 hours before the X-ray contrast reached the distal ileum

Finally after pretreatment with phthalylsulphathiazole, oral histamine was still converted to urinary conjugated histamine, although on a smaller scale A bacterial origin of the conjugated histamine in this subject cannot be entirely ruled out, since the bacteria were not necessarily eradicated, although they probably were substantially reduced.

It would appear that measurement of urinary N-acetylhistamine is not a method for evaluating the degree of absorption of unchanged histamine. Most of the radioactivity can eventually be recovered from the urine when ^{14}C-histamine is ingested (SCHAYER 1956) Methylimidazole-acetic acid seems to be the greatest urine metabolite (64 %), as after subcutaneous injection (SCHAYER 1956 SCHAYER & COOPER 1956)

It is feasible that acetylation is of importance in the catabolism of oral histamine only when large doses are given. A marked rise in urinary conjugated histamine followed 18 mg of oral histamine. With smaller doses, however an increment in urinary conjugated histamine could easily be masked In order to establish whether small doses of oral histamine occasion extra urinary N acetylhistamine excretion, isotopic technique will have to be applied. In his experiment, SCHAYER (1956) did not examine whether ^{14}C N-acetylhistamine was present in the urine after oral ^{14}C histamine.

Dietary N-acetylhistamine and dietary histamine could be responsible for part of the normal urinary conjugated histamine, provided conjugation is also a catabolic pathway for small quantities of intestinal histamine. The effect of fairly small quantities of histamine on intestinal motility would probably be minor Histamine and N-acetylhistamine would probably be absorbed before gaining access to segments with abundant resident flora. *In vitro* studies on rats and guinea pigs have demonstrated that intestinal mucosa and liver tissue have a capacity for acetylating sulphonamides (HARTIALA & TERHO 1965) The latter finding may have a bearing on man as well. Sulphonamides and histamine apparently require the same enzyme system for acetylation (TABOR 1956).

It thus seems likely that conjugated histamine, normally occurring in urine, in part comes from dietary N-acetylhistamine and histamine. The acetylation of histamine may in part take place extraluminally When

larger amounts of histamine are ingested, intraluminal acetylation is superposed the higher the dose, the higher the percentage gaining access to the colon and the higher the percentage of N-acetylhistamine in the urine.

Summary

1 Histamine and N-acetylhistamine were administered orally and rectally to healthy individuals, as well as to various segments of the gut during gynaecological surgery Free and conjugated histamine were measured in urine and faeces.

2. A marked increase in urinary conjugated histamine occurred, without a concurrent rise in free histamine, regardless of the site of histamine administration. In particular a high output was found after administering histamine into the colon. A fairly close correlation (r = +0 70) was found between the oral dose of histamine and the extent of urinary N acetylhistamine excretion. Faecal excretion of free histamine-like activity as a percentage of the dose administered, tended to rise with increasing doses of oral histamine.

3 On average, 77 / of oral N acetylhistamine was excreted as such in the urine N-acetylhistamine instilled rectally is changed intraluminally and the free variety appears in the lumen No clear increase in urinary free histamine occurred after N acetylhistamine administration.

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From the Department of Pharmacology University of Göteborg, Sweden

The Action of Triiodothyronine on Some Effects of Adrenaline and Noradrenaline in Man.

By

Nils Svedmyr

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In experiments on rabbits I have previously reported that thyroxine (T_4) medication, at a dose that increased basal metabolism by 45–65 %, potentiated the calorigenic effect of adrenaline The stimulatory effect of adrenaline on lactic acid production was also potentiated, but no increase was seen in its effect on blood glucose concentration or on the concentration of free fatty acids in the plasma. The potentiation of the calorigenic effect could largely be ascribed to an increase in lactic acid production and metabolism. After thyroidectomy the calorigenic and hyperlactacidemic effects of adrenaline were diminished. T_4 medication did not potentiate the calorigenic effect of noradrenaline or its influence on lactate, glucose or free fatty acids concentration in the blood (SVEDMYR 1966a, b)

Also in man thyroid hormone has been considered of importance for the calorigenic action of adrenaline. The calorigenic effect of adrenaline is most marked in hyperthyroid patients (HORSTMAN 1954 MURRAY & KELLY 1959) and weakest in those with hypothyroidism (HORSTMAN 1954)

The mechanism of the calorigenic effect of adrenaline in man appears to be more complicated than in the rabbit. In previous experiments (SVEDMYR 1966c) it was found that an increase in both lactic acid and free fatty acids metabolism was of importance for the calorigenic effect of adrenaline, and it was in these tests estimated that the increased free fatty acids mobilization contributed about 30 % and the lactic acid mobilization to about 35 % of the total calorigenic effect. For the remainder it was considered that, among other factors, stimulation of the work of the heart was of some importance. The hyperglycemic effect of adrenaline is probably also of some, if only of relatively limited, importance (BOOTHBY & SANDIFORD 1923) It is therefore plausible *a priori* to hold that potentiation of the calorigenic effect of A by thyroid hormone in man may take place partly by means of the mechanisms mentioned above. The effect of

adrenaline and noradrenaline infusions on oxygen consumption, on blood lactate and glucose concentrations, on the concentration of free fatty acids in plasma and on certain circulatory variables were therefore studied before and during treatment with triiodothyronine.

Noradrenaline exerts practically no effect on lactic acid production in man (LUNDHOLM & SVEDMYR 1966) nor does it stimulate the pulse frequency or cardiac minute volume (BARCROFT & SWAN 1953) on the other hand its free fatty acids-mobilizing effect is equal to or stronger than that of adrenaline By also studying the way in which triiodothyronine medication affects the calorigenic and free fatty acids-mobilizing actions of noradrenaline, it should be possible to determine more selectively the role of free fatty acids mobilization in the potentiation of the calorigenic effect of the catecholamines by thyroid hormone. Since it was found that in man the effect of adrenaline on the blood lactate concentration was augmented after treatment with triiodothyronine, the metabolism of infused L(+)lactate was also studied before and during triiodothyronine medication These experiments are reported elsewhere (SVEDMYR 1966d).

It is also conceivable that a potentiated adrenaline effect might be due to reduced inactivation of adrenaline (review HARRISON 1964). The plasma adrenaline concentration was therefore determined during the adrenaline infusion, and the effects of treatment with thyroid hormone were studied. These experiments are also reported elsewhere (HÄGGENDAL & SVEDMYR 1966) Further details of the relationship between the physiological and pharmacological effects of the catecholamines and of the thyroid hormones have been discussed in reviews by ELLIS (1956), HOCH (1962), HARRISON (1964), ROSENBERG & BASTOMSKY (1965) LUNDHOLM, MOHME-LUNDHOLM & SVEDMYR (1966)

Method

The experiments were performed on two groups of healthy male subjects, aged 22–39 years, accustomed to such investigations. The first group consisted of 6 persons, on whom, first, the effect of adrenaline infusion on different functions was studied under basal condition (control experiments). The same subjects were then given 1 mg triiodothyronine orally under supervision, and 2–5 days later the adrenaline test was repeated. The adrenaline effect before and after the triiodothyronine treatment was then compared in each subject. The second group consisted of 5 subjects, one of whom was also included in the first group. This second group underwent the same investigation with the exception that noradrenaline was infused instead of adrenaline and that the second noradrenaline tests were made exactly 2 days after administration of triiodothyronine.

The subject came to the laboratory in the morning, having fasted since the previous evening. He lay on an examination table in a room thermostatically controlled at 24.0 ± 0.5°C. A plastic catheter was introduced into a cubital vein in each arm, one for the withdrawal of blood samples (via a three way tap) and the other for infusion of adrenaline or

noradrenaline. When the catheters were not being used for these purposes, 0.9 % NaCl solution without the addition of heparin was infused into each of them. After inserting the catheters and beginning NaCl infusion, the subject rested for 60 minutes, then the basal metabolism, blood pressure and pulse were measured during two 10-mi utes periods. A basal blood sample was taken during each f these periods, and their mean values were taken as initial values. The infusio solution was then changed in the first series to drenaline, which was given in dose of 0.10 μg/kg/min f 30 minutes, dissolved in 0.9 % NaCl solution to which 0.1 % ascorbic acid had been added to prevent xidation. The metabolism was subsequently determined during the periods 0–10, 25–35 55–65, 85–95 and 115–125 minutes after beginning drenaline infusio Blood samples (18 ml) were taken 10, 20, 30, 60, 90 and 120 minutes after beginning A infusion, for determination of lactic acid, glucose, free fatty acids and catecholamines. In th second series, with noradrenaline infusions, the blood catecholamine concentration was not determined, and therefore only 8 ml of blood were withdrawn each time. In this series the metabolism was also measured during the period 43–48 minutes fter beginning infusion, nd further blood sample was taken after 45 minutes. Otherwise the experiments were performed in the same way in the two series. The blood pressure was measured by auscultation i all experiments, t 5–10 minute intervals, and the pulse was also recorded at similar intervals. The O_2 consumption, ventilation and CO_2 elimination were measured with Hartman and Braun metabolic recorder and were recorded with a Grass polygraph, which has been described previously (SVEDMYR 1966c) The body temperature was recorded in some tests. The maximal increase was small (0 15°C) in all groups.

The blood lactate concentration was determined enzymatically by the method f LUNDHOLM, MOHME-LUNDHOLM & VAMOS (1963), the blood glucose by that f BERGMEYER & BERNT (1962) and the plasma free fatty acid concentration by that of TROUT ESTES & FRIEDBERG (1960).

Results.

Effect of oral administration of 1 mg triiodothyronine on basal metabolism and circulation The subjects stated that they had pronounced tachycardia and anxiety even on the day after taking triiodothyronine. On examination 2.5 or 2 days later distinct thyrotoxic symptoms with palpitations, raised pulse pressure, slight tremor and nervousness, were noted. As shown in table I there were statistically significant increases in O_2 consumption, CO_2 elimination and pulse rate The systolic blood pressure rose some what, but the diastolic blood pressure and the blood lactate, glucose or free fatty acids concentrations were not affected unequivocally

The triiodotyronine dose was chosen in consideration of the further pulse increase which was to be expected during the adrenaline infusion. This dose allowed the subject to lie relaxed during the infusion, but neverthe less gave a statistically certain potentiation of the effect of adrenaline on O_2 consumption.

Adrenaline effect before and during triiodothyronine medication

O_2 consumption. The results are given in table II and figs. 1 A and 2. In view of the fact that triiodothyronine affected the basal O_2 consumption, the stimulatory effect of adrenaline has not been expressed as a percent

age of the basal value but as the increase in ml O_2/kg/min. over the basal value. The oxygen consumption began to increase soon after beginning the adrenaline infusion and reached half its maximal values after 5 minutes. The maximum was attained at the end of the infusion, and not until 60 minutes later was the basal value regained. After triiodothyronine medication the stimulatory effect of adrenaline on O_2 consumption was greater throughout than under basal conditions. This potentiation was observed in all subjects and was statistically significant. When calculated from fig. 1 A for the period 0–90 minutes the potentiation of the adrenaline effect was 85 /

CO_2 elimination. Under basal conditions, CO_2 elimination was increased to a greater extent than O_2 consumption during the adrenaline infusion (table II) The probable reason for this finding has been discussed elsewhere (LUNDHOLM & SVEDMYR 1966) The effect of adrenaline on CO_2 elimination was potentiated after triiodothyronine medication (fig. 2, table II)

Ventilation. During adrenaline infusion the time course of the ventila-

Table I

The effect of 1 mg triiodothyronine (T_3) on the basal metabolism d some other functions in man. Mean increase ± S.E.M. over basal values in the same persons. P = probability that the effect was due to chance.

	Adrenaline series (n = 6)		Noradrenaline series (n = 5)	
	Basal value	Mean change after T_3 treatment	Basal value	Mean change after T_3 treatment
O_2 consumption ml/kg/min.	3 66 ± 0.063	0.51 ± 0.057 P < 0 001	3 43 ± 0.137	0.75 ± 0.107 P < 0 005
Ventilation l/min.	5 61 ± 0.262	0 87 ± 0.170 P < 0.005	5 91 ± 0.219	0.81 ± 0.239 P < 0 05
CO_2 elimination ml/kg/min	2.85 ± 0 066	0.42 ± 0.084 P < 0.005	2.74 ± 0.136	0 47 ± 0 01 P < 0 001
Lactic acid mg/100 ml	5 7 ± 0.35	−0.1 ± 0.36	5 4 ± 0.074	−0 6 ± 0 51
Glucose mg/100 ml	68 ± 3 2	5 ± 6.2	76 ± 2.5	1 ± 5.4
Free fatty acids µeq/l	740 ± 31	20 ± 49	790 ± 29	43 ± 95
Pulse rate/min	53 ± 0.6	16 ± 2.2 P < 0 001	54 ± 4.8	17 ± 2.5 P < 0 005
Systolic B.P mm Hg	114 ± 2.1	12 ± 2.0 P < 0.005	119 ± 1.0	7 ± 3 1
Diastolic B.P mm Hg	74 ± 1.3	3 ± 1.7	81 ± 2.2	5 ± 2.0

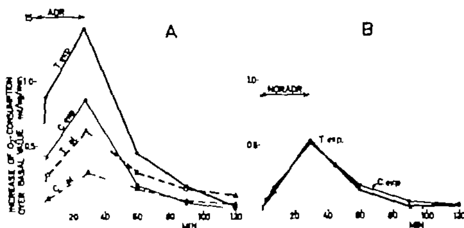


Fig. 1

A. Effect f adrenaline infusion (0.10 μg/kg/min. fo 30 min.) on the oxygen consumption of untreated (C exp) and triiodothyronine-treated (T exp) subjects. Mean of 6 experiments. T cal and C cal = expected increase f oxygen consumption in the same experiments, calculated from the increased lactate content of the blood.

B. The effect of noradrenaline infusion (0.15 μg/kg/min. fo 30 min.) on the xygen consumptio of untreated (C exp) and triiodothyronine-treated subjects (T exp). Mean of 5 experiments.

tion closely followed that of the CO_2 elimination Triiodothyronine medication resulted in a tendency to potentiate the respiration stimulating effect of adrenaline, and the ventilation and CO_2 elimination curves still ran parallel (fig. 2, table II)

Blood lactate concentration Under basal conditions adrenaline increased the blood lactate concentration by a maximum of 9 0 mg / (fig. 2, table II). In all tests triiodothyronine medication potentiated this hyperlactacidemic effect of A (the increase being found statistically significant.)

Blood glucose concentration The blood glucose concentration increased significantly during the adrenaline infusion (fig. 2, table II) the effect was unaltered by triiodothyronine medication

Plasma free fatty acid concentration. Under basal conditions the plasma concentration of free fatty acid increased during the adrenaline infusion (fig. 2, table II). After triiodothyronine medication, which did not affect the basal free fatty acid concentration, the increase was somewhat smaller than under basal conditions. This reduction of the adrenaline effect was statistically probable when calculated on the sum of the differences for 10–60 minutes. The mean difference was 127 ± 50.2 ($P < 0.05$)

Circulation. The stimulatory effect of adrenaline on the pulse rate was not potentiated to a statistically significant extent by triiodothyronine

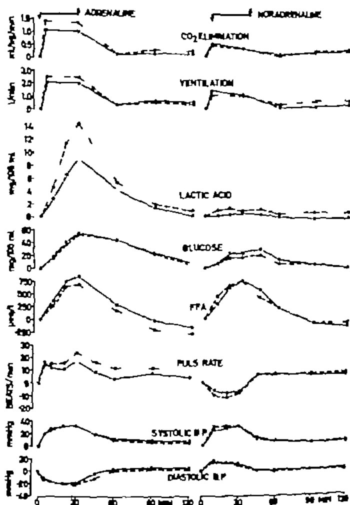


Fig. 2. The effect of adrenaline and noradrenaline infusions on some metabolic and circulatory functions in man. Continuous lines ●—●—●—● = experiments on untreated subjects. Broken lines ○ ○–○ = experiments on triiodothyronine-treated subjects. Adrenaline experiments mean of 6 tests. Noradrenaline experiments mean of 5 tests.

medication. However during the period 10–120 minutes after beginning the adrenaline infusion there was a tendency towards a greater pulse increase after the triiodothyronine treatment than under basal conditions (fig. 2). Triiodothyronine medication had no effect on the action of adrenaline on systolic or diastolic blood pressure (fig. 2).

Effect of noradrenaline before and after triiodothyronine medication

The effect of noradrenaline on the different variables is shown in figs. 1 B and 2 and in table III. The increase in O_2 consumption during the noradrenaline infusion began later than during adrenaline infusion, and

Table 2

Effect of adrenaline infusion (0.1 μg/kg/min. for 30 min.) on some metabolic and circulatory functions in man.

Tests before (A) and after triiodothyronine (AT_3) treatment. I the triiodothyronine tests the mean increase in the drenaline effects before and after triiodothyronine treatment in the same person have been calculated (AT_3-A). The times refer to the beginning of the adrenaline infusion. Mean of 6 tests. P = probability that the effect was due to chance. Significant levels [1] = $P < 0.05$ [2] = $P < 0.01$ [3] = $P < 0.001$

		A Mean increase over basal value	AT_3 Mean increase over basal valu	AT_3-A
O_2 consumption ml/kg/min.	0–10 min.	0.41 ± 0.056[3]	0.86 ± 0.095[3]	0.45 ± 0.093[3]
	25–35	0.85 ± 0.061[3]	1.41 ± 0.134[3]	0.56 ± 0.104[3]
	55–65 –	0.20 ± 0.051[1]	0.44 ± 0.102[1]	0.24 ± 0.125
CO_2 elimination ml/kg/min.	0–10	1.09 ± 0.105[3]	1.40 ± 0.184[3]	0.31 ± 0.096[3]
	25–35	1.00 ± 0.125[3]	1.31 ± 0.168[3]	0.31 ± 0.119[1]
	55–65 –	0.07 ± 0.45	0.10 ± 0.083	0.03 ± 0.062
Ventilation l/min.	0–10	2.06 ± 0.219[3]	2.47 ± 0.351[3]	0.42 ± 0.181
	25–35	1.97 ± 0.189[3]	2.42 ± 0.315[3]	0.45 ± 0.189
	55–65 –	0.3. ± 0.110[1]	0.17 ± 0.114	−0.15 ± 0.178
Lactic acid in blood mg/100 ml	10	2.2 ± 0.73[1]	4.5 ± 1.11[2]	2.3 ± 0.70[1]
	20	6.4 ± 1.37[2]	11.3 ± 0.99[3]	4.9 ± 0.60[3]
	30 –	9.0 ± 3.00[1]	13.3 ± 1.40[3]	4.4 ± 0.67[2]
	60 –	4.1 ± 1.17[1]	5.2 ± 1.10[2]	1.1 ± 0.91
Blood glucose mg/100 ml	10	17 ± 7	14 ± 1.4[2]	−3 ± 8
	20 –	40 ± 10[1]	36 ± 12.5)	−5 ± 7
	30	55 ± 6[2]	52 ± 13.5)	−2 ± 12
	60	44 ± 5[2]	43 ± 10[2]	−1 ± 9
Free fatty acids in plasma μeq/l	10	372 ± 83.5[2]	263 ± 77.7[1]	−109 ± 54.9
	20	736 ± 91.0[3]	63 ± 97.9[2]	−104 ± 97.1
	30	852 ± 90.8[3]	684 ± 115.1[2]	−168 ± 142.3
	60 –	300 ± 95.2[1]	172 ± 35.8[2]	−128 ± 115.1
Pulse rate/min	10 –	13 ± 2.3[2]	15 ± 2.8[2]	3 ± 2.1
	30	18 ± 1.9[3]	4 ± 6.0[2]	7 ± 5.2
	60	4 ± 1.3[1]	13 ± 4.5)	9 ± 4.3

		A Mean increase over basal value	AT_3 Mean increase over basal value	AT_3-A
Systolic B.P. mm Hg	10 –	27 ± 3.9[3]	24 ± 3.5[3]	−3 ± 2.0
	30 –	32 ± 4.4[3]	31 ± 2.3[3]	−1 ± 3.7
	60 –	10 ± 2.0[3]	11 ± 2.7[3]	1 ± 3.0
Diastolic B.P. mm Hg	10 –	−16 ± 2.2[3]	−17 ± 1.3[3]	−2 ± 2.4
	30 –	−18 ± 2.5[3]	−22 ± 2.7[3]	−4 ± 2.6
	60 –	4 ± 1.5	1 ± 1.1	−3 ± 2.0

Table 3

Effect of noradrenaline (NA) infusion (0.15 μg/kg/min. for 30 min.) on some metabolic and circulatory functions in man. Notations as in Table II.

		NA Mean increase over basal value	NAT_3 Mean increase over basal value	NAT_3-NA
O_2 consumption ml/kg/min.	0–10 min.	0.11 ± 0.031[1]	0.09 ± 0.059	−0.02 ± 0.059
	25–35 –	0.52 ± 0.032[3]	0.53 ± 0.057[3]	+0.01 ± 0.073
	55–65 –	0.17 ± 0.020[3]	0.14 ± 0.053	−0.03 ± 0.071
CO_2 eliminatio ml/kg/min.	0–10 –	0.47 ± 0.041[3]	0.35 ± 0.124[1]	−0.12 ± 0.113
	25–35 –	0.25 ± 0.090[1]	0.25 ± 0.082[1]	0.00 ± 0.042
	55–65	0.02 ± 0.031	−0.06 ± 0.054	−0.09 ± 0.084
Ventilation l/min.	0–10	1.41 ± 0.218[2]	1.00 ± 0.297[1]	−0.41 ± 0.410
	25–35 –	1.12 ± 0.155[3]	0.93 ± 0.168[1]	−0.19 ± 0.230
	55–65 –	0.07 ± 0.189	0.15 ± 0.280	0.08 ± 0.165
Lactic acid in blood mg/100 ml	10 –	−0.1 ± 0.64	0.9 ± 0.30[1]	0.9 ± 0.50
	20 –	0.2 ± 0.50	1.0 ± 0.48	0.9 ± 0.45
	30 –	0.3 ± 0.48	0.6 ± 0.43	0.4 ± 0.45
	60 –	−0.3 ± 0.33	0.1 ± 0.57	0.4 ± 0.73

		NA Mean increase over basal value	NAT_3 Mean increase over basal value	NAT_3-NA
Glucose in blood mg/100 ml.	10 –	7 ± 10.0	7 ± 1.5[1])	0 ± 9.3
	20 –	21 ± 6.6[1])	15 ± 3.1[2])	−6 ± 7.4
	30 –	23 ± 11.6	16 ± 3.6[1])	−7 ± 11.5
	60 –	12 ± 8.0	5 ± 1.1[1])	−7 ± 7.6
Free fatty acids in plasma μeq/l	10 –	298 ± 61.2[2])	442 ± 160.0[1])	144 ± 111
	20 –	600 ± 148.0[1])	649 ± 152.2[1])	48 ± 146
	30 –	749 ± 124.1[2])	745 ± 216.0[1])	−5 ± 180
	60 –	169 ± 163.0	188 ± 179.1	19 ± 125
Pulse rate/min.	10	−6.0 ± 1.9[1])	−10.2 ± 2.2[2])	−4.2 ± 2.9
	30 –	−7.4 ± 2.1)	−9.4 ± 1.4[2])	−2.0 ± 2.8
	60 –	+5.8 ± 1.3[1])	+5.2 ± 4.5	−0.6 ± 3.6
Systolic B.P. mm Hg	10 –	24 ± 5.4[1])	29 ± 3.7[2])	5 ± 5.0
	30 –	32 ± 4.6[2])	30 ± 2.5[3])	−2 ± 5.[illegible]
	60 –	4 ± 1.7	5 ± 1.6[1])	1 ± 2.3
Diastolic B.P. mm Hg	10	16 ± 1.5[2])	12 ± 2.9[1])	−3 ± 2.3
	30	12 ± 1.8[2])	9 ± 3.0[3])	−3 ± 4.3
	60	−1 ± 1.1	0 ± 1.6	0.4 ± 1.3

its total effect during the period 0–65 minutes was about 55% of the adrenaline effect. The effect of noradrenaline on the ventilation and CO_2 elimination was also less than that of adrenaline. In contrast to adrenaline, noradrenaline showed only a small effect on blood lactate concentration. The hyperglycemic effect of noradrenaline was approximately half that of adrenaline, and the effect on plasma concentration of free fatty acids was the same as that of adrenaline.

In contrast to adrenaline, noradrenaline reduced the pulse rate and raised the diastolic blood pressure, whereas the effect on the systolic blood pressure was the same for both catecholamines. Treatment with triiodothyronine did not alter the calorigenic effect of NA, but its effect on blood lactate concentration was possibly somewhat augmented. Otherwise the triiodothyronine medication caused no alteration to any metabolic or circulatory effect of noradrenaline

Discussion

In these experiments triiodothyronine medication had a significant, even if not a particularly powerful effect, on basal metabolism and the circulation. The stimulatory effect of adrenaline on oxygen consumption increased by 85% after triiodothyronine treatment, and its effect on the blood lactate concentration was also potentiated. The hyperglycemic effect was not affected by the triiodothyronine medication and its effect on the plasma concentration of free fatty acids appeared to be reduced. The action of adrenaline on blood pressure and pulse rate was not affected unequivocally by the triiodothyronine treatment.

In previous experiments on man (SVEDMYR 1966c) the proportion of the total calorigenic effect of adrenaline that could be ascribed to the stimulation of lactic acid metabolism was estimated as 35%. The potentiation of the effect of adrenaline on the blood lactate concentration after triiodothyronine medication could either be due to the fact that the hyper

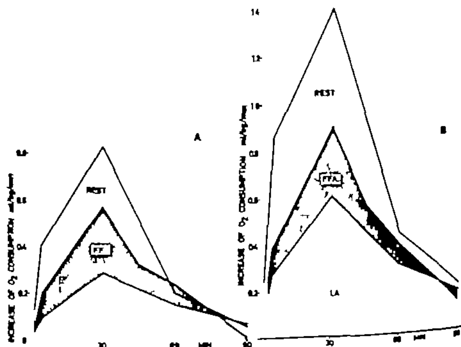


Fig. 3. Tentative presentation of the relative importance of lactic acid (L.A.) and metabolism of free fatty acids for the calorigenic effect of adrenaline in untreated (A) and triiodothyronine-treated subjects (B). L.A. increase in oxygen consumption calculated from the relationship between lactic acid concentration in the blood and stimulation of oxygen consumption, as found by Svedmyr (1966d). FFA proportion of the calorigenic effect of adrenaline blocked by nicotinic acid and attributed to increased metabolism of free fatty acids (Svedmyr 1966c)

lactacidemic effect of adrenaline had been potentiated or that the rate of lactic acid elimination had been reduced. In complementary experiments (SVEDMYR 1966d) it was found, however that after triiodothyronine medication infused L(+)lactate in man was metabolized more rapidly than in untreated subjects. The increased effect of adrenaline on the blood lactate concentration after triiodothyronine medication was therefore due to an increase in lactic acid production. On infusion of L(+)lactate before and during treatment with triiodothyronine the regression equations given below were obtained for the relation between the stimulation of oxygen consumption and the increase in blood lactate concentration (SVEDMYR 1966d) Before triiodothyronine treatment $Y = 0.028 X + 0.032$ after triiodothyronine treatment $Y = 0.038 X + 0.099$ (Y = increase of oxygen consumption in ml/kg/min. X = increase of blood lactate concentration in mg%) On calculating by means of these equations the "expected" increase in oxygen consumption that should be induced by the increased lactate concentration in the blood during the adrenaline infusion, the curve L. A. in fig. 3 was obtained. The area below this curve during the period 0–90 minutes was 40% of the total adrenaline effect before and 49% after triiodothyronine medication. The importance of the lactic acid metabolism appeared therefore to increase after triiodothyronine administration. Triiodothyronine increased the calorigenic effect of adrenaline by 85% and of this increase 58% could probably be ascribed to potentiation of lactic acid metabolism.

It is very probable, that increased lactic acid metabolism only partly explains the potentiating effect of thyroid hormones on the calorigenic action of adrenaline and that some further mechanism or mechanisms also play a part.

In untreated subjects, approximately 30% of the calorigenic effect of A could be ascribed to an increase in metabolism of free fatty acids (SVEDMYR 1966c) (fig. 3) An important question is whether after triiodothyronine treatment the role of mobilization of free fatty acids in the calorigenic effect of adrenaline had also increased. The elevating effect of adrenaline on the plasma concentration of free fatty acids was somewhat smaller after triiodothyronine treatment than in untreated subjects. This may clearly have been due either to a decrease in mobilization of free fatty acids or to an increase in metabolism of free fatty acids. On the other hand, neither the free fatty acid-mobilizing nor the calorigenic action of noradrenaline was affected by triiodothyronine treatment. It seems improbable that triiodothyronine would affect by substantially different pathways the free fatty acids-mobilizing activities of adrenaline and noradrenaline and the part played by free fatty acids-mobilization in the calorigenic effect. In fig. 3 the actual increase in oxygen consumption due

to increased mobilization of free fatty acids has been assumed to be the same in triiodothyronine-treated and untreated subjects.

Some investigators have found, however that the free-fatty-acids-mobilizing effects of catecholamines are potentiated by thyroid hormones. This was demonstrated in the rat (DEBONS & SCHWARTZ 1961 DEYKIN & VAUGHAN 1963), but not confirmed in the rabbit (SVEDMYR 1966a). HARLAN *et al* (1962) found in man that the free-fatty-acids-mobilizing effects of both adrenaline and noradrenaline were potentiated in hyperthyroidism and reduced in hypothyroidism, but HAMBURGER *et al.* (1963) were unable to verify these findings.

The reason for this disagreement is not clear It is conceivable that both differences between species and possibly the stronger effect of thyroid hormone may have been of importance when potentiation of the free-fatty-acids mobilizing effects of catecholamines have been demonstrated. It is probable that, if the free-fatty acids-mobilizing action of catecholamines had been potentiated by thyroid hormone, this action would have contributed to potentiation of the calorigenic effect. Further studies of this problem are required.

Under the experimental conditions used, stimulation of the hyperlactacidemic effect of adrenaline and the potentiation of lactic acid metabolism are probably among the more important mechanisms by which thyroid hormone potentiates the calorigenic effect of adrenaline.

The effect of adrenaline on ventilation and CO_2 elimination was also augmented after triiodothyronine medication. The stimulatory effect of adrenaline on ventilation is due, at least in part, to an increase in CO_2 production This, in turn is due to an increase in oxygen consumption and in lactic acid production in the tissues. (LUNDHOLM & SVEDMYR 1966) It seems plausible, therefore, to ascribe the augmented stimulation of ventilation and CO_2 elimination to the potentiating influence of triiodothyronine on the adrenaline effect on lactic acid production and O_2 consumption

Triiodothyronine medication did not alter the plasma adrenaline concentration during the adrenaline infusion (HÄGGENDAL & SVEDMYR 1966) The observed potentiation of certain adrenaline effects does not therefore appear to be ascribable to changes in adrenaline metabolism.

Summary

A study was made on human subjects of the effect of triiodothyronine medication on the calorigenic actions of adrenaline and noradrenaline and on their effects on the blood lactate and glucose concentrations and the concentration of free fatty acids in plasma. Triiodothyronine medica-

tion potentiated the calorigenic and hyperlactacidemic effects of adrenaline whereas its effect on blood glucose concentrations was not altered and its effect on the concentrations of free fatty acids in the plasma was somewhat diminished. The circulatory effect of adrenaline were not affected unequivocally by the triiodothyronine treatment. Triiodothyronine did not potentiate either the calorigenic or free fatty acids-mobilizing effects of noradrenaline, nor were any of the other noradrenaline effects unequivocally modified An increase in metabolism of free fatty acids was therefore probably of minor importance for the potentiation of the calorigenic effects of adrenaline by triiodothyronine in these experiments. It was estimated that increased lactic acid metabolism contributed to 40 / of the calorigenic effect of adrenaline before and 49 / after treatment with triiodothyronine. The potentiated effect of adrenaline on the lactate metabolism after triiodothyronine administration could therefore partly explain the increase in the calorigenic effect.

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From the Department of Pharmacology Univ of Lund, Lund, Sweden

Radiometric Micromethods for the Study of Some Amino Acid Decarboxylases

By

Rolf Håkanson

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The classical method for the study of amino acid decarboxylase activity is manometric and is based on the enzymatic production of carbon dioxide. Determination of the amount of amine produced became possible when spectrophotometric and fluorometric methods (BOGDANSKI, PLETSCHER, BRODIE & UDENFRIEND 1956 WAALKES & UDENFRIEND 1957 LOWE, ROBINS & EYERMAN 1958 HESS & UDENFRIEND 1959 SHORE, BURKHALTER & COHN 1959 CARLSSON & WALDECK 1959) were introduced Fluorometric techniques are sensitive enough for most purposes, but are inadequate for studies of the low enzyme activities found in most isolated cells or small samples of tissue Labelled substrates, however have made such studies possible (SCHAYER 1956 KAHLSON, ROSENGREN, WESTLING & WHITE 1958 ALBERS & BRADY 1959 WEISSBACH, KING, SJOERDSMA & UDENFRIEND 1959 WILSON, GIBSON & UDENFRIEND 1960 KOBAYASHI 1963 SNYDER & AXELROD 1964 MCCAMAN, MCCAMAN HUNT & SMITH 1964), but most of the techniques are laborious and time-consuming. The growing interest in the function and formation of biogenic amines has stimulated the development of simple, rapid methods for measuring low activities of amino acid decarboxylases. This paper describes several such techniques. Some of them have been described, with minor modifications, in previously published studies of amino acid decarboxylase activities in biological material (HÅKANSON 1963 HÅKANSON & MÖLLER 1963 HÅKANSON 1964 ENEMAR, FALCK & HÅKANSON 1965 HÅKANSON MÖLLER & STORMBY 1965 HÅKANSON & OWMAN 1965).

Material and Methods

The technical device for ion exchange chromatography developed by BERTLER, CARLSSON & ROSENGREN (1958) was employed throughout. Before use, the columns, containing 100 mg (dry weight) Dowex 50–X4 (200-mesh, column dimensions 13 mm² × 20 mm), were washed alternately several times with 4 N hydrochloric acid and redistilled water. The flow rate was kept at about 0.25 ml per minute.

All radioactive chemicals were obtained from The Radiochemical Centre, England, with the exception of ^{14}C-γ-amino-n-butyric acid, which came from the New England Nuclear Corp., USA. The labelled compounds were stored at −20° before and after purification. Most measurements of radioactivity were carried out in a windowless Frieseke-Hoepfner gas flow-counter with an instrumental background value of about 20 counts per minute (cpm). At least 4000 counts were taken for each sample. The background count was subtracted from the observed total count.

DL 3(3 4 Dihydroxyphenyl)-alanine-2-^{14}C (*DOPA*) had a specific activity of 3.75 mc/mM and gave 3.5 × 10^4 cpm/μg at zero thickness. Before use, the ^{14}C-DOPA preparation was purified on a Dowex 50 column (HÅKANSON & MÖLLER 1963). The radioactive DL DOPA was dissolved in redistilled water and transferred to a 300 mg Dowex 50 column, previously equilibrated with 20 ml 4 N hydrochloric acid and rinsed with 5 ml redistilled water. After passage of the solution, the column was washed with 30 ml 0.5 M sodium acetate-acetic acid buffer pH 3.5. Elution of the amino acid, which was retained on the column, was performed with 0.4 M acetate buffer pH 6.0, all DOPA appearing in the second to fourth ml. The eluate was diluted with 0.01 N hydrochloric acid to the desired concentration. Interfering radioactive impurities of unknown identity were removed by this procedure.

^{14}C-L-Histidine labelled in the position in the imidazole ring, had a specific activity of 1.9 mc/mM and gave 14 × 10^4 cpm/μg at zero thickness. Since the commercial preparation contained small amounts of radioactive histamine (WHITE 1960), it was purified by passage through the standard Dowex 50 column. The radioactive histidine was dissolved in 1 ml 0.1 M phosphate buffer pH 8, and the volume was adjusted to 5 ml by means of redistilled water. This solution was transferred to the column, previously treated with 10 ml 0.8 M phosphate buffer pH 8.0, and 5 ml redistilled water. Histidine passed through in the effluent, histamine being retained on the column. Approximately 80 % of the histidine could be recovered in the effluent the remaining 20 % was eluted by washing the column with 5 ml 0.03 M phosphate buffer pH 8. The combined histidine-containing fractions were evaporated to dryness on a steam bath, and the dry residue was dissolved in 0.01 N hydrochloric acid to the desired volume. All histamine was eliminated by this procedure. The final preparation still contained some unidentified radioactive impurity that could be shown by paper chromatography (HÅKANSON, unpublished result), but this impurity did not interfere with the experiments (fig. 2).

L-Glutamic acid-^{14}C (uniformly labelled) mono-ammonium salt, had specific activity of 27 mc/mM (13.5 × 10^4 cpm/μg at zero thickness). Before use the commercial preparation was purified on a Dowex 50 column, equilibrated as described below (in Measurement of glutamic acid decarboxylase activity). The ^{14}C-glutamic acid was dissolved in redistilled water and passed through the column. Most of the amino acid was recovered in the effluent, and the rest was eluted by few ml of 0.1 M ammonium acetate-acetic acid buffer pH 3.5. The combined fractions containing glutamic acid were evaporated to dryness on a steam bath, and the dry residue was dissolved in 0.01 N hydrochloric acid to the desired volume. Interfering radioactive impurities of unknown identity were removed by this procedure.

3-(3 4 Dihydroxyphenyl)-ethylamine-1-^{14}C (*dopamine*) with specific activity of 3.75 mc/mM was obtained by incubating ^{14}C-DL DOPA with the supernatant of rabbit kidney

homogenate (*cf* ROSENGREN 1960). The ^{14}C-dopamine formed was isolated by the method described below and taken up in 0.01 N hydrochloric acid. T establish the yield of ^{14}C dopamine, the kidney extract was similarly incubated with the same amount of non-radioactive DL-DOPA and the formation of dopamine was determined fluorometrically as described by CARLSSON & WALDECK (1958).

Histamine-2(ring)-^{14}C dihydrochloride had a specific activity of 21.3 mc/mM (19 × 10^4 cpm/μg base at zero thickness) and was dissolved in 0.01 N hydrochloric acid before use.

γ-Amino-n-butyric-1-^{14}C acid (GABA) had specific activity of 3.72 mc/mM (3.6 × 10^4 cpm/μg at zero thickness) and was dissolved in redistilled water before use.

Enzyme activity assays were performed on isolated cells, small amounts of tissue not exceeding 100 mg wet weight or tissue extracts. A metabolic incubator equipped with a shaking device was used. All incubations were performed in 0.1 M phosphate buffer (Sörensen) in nitrogen atmosphere at 37°. Blank values were btained by parallel identical incubations without tissue or with heated tissue samples (tissue blanks). Blank values were subtracted from the figures indicating enzyme activity. Standards were provided by running known amounts of ^{14}C-labelled amines simultaneously. As a further control, i some experiments known amounts f the ^{14}C-labelled amines were added to one or two samples in series (internal standards).

Measurements of DOPA decarboxylase activity

The sample was incubated with 3 μg ^{14}C DL DOPA in a total volume of about one ml for one hour. Incubation was interrupted by the addition of 4 ml 0.4 N perchloric acid containing 10 μg nonradioactive dopamine as carrier. After homogenization in an Ultra Turrax homogenizer the precipitate was spun down, and the supernatant was neutralized with 2 N potassium carbonate to a final pH of about 6.5 and centrifuged. The subsequent isolation of the amine was performed essentially as described by BERTLER *et al* (1958). The supernatant was transferred to the Dowex 50 column, previously treated with 10 ml M sodium acetate-acetic acid buffer pH 6.5, and 5 ml redistilled water. After passage of the sample, the column was washed with 35 ml 0.06 M phosphate buffer pH 6.6, and 10 ml redistilled water. All DOPA was eliminated from the column by that procedure, and the amine was then quantitatively eluted with 10 ml N hydrochloric acid. The eluate was evaporated to dryness on steam bath, during which procedure the heat-labile catechol amines were destroyed. The dry residue was extracted twice with 3 ml acetone containing 0.1 ml 2 N hydrochloric acid per 100 ml. Radioactivity of the samples was quantitatively recovered in the acetone extract, which was then transferred to standard planchettes, diameter one inch, and evaporated to dryness for measurement of radioactivity. Besides the amines the eluate contained some radioactive impurities originating from the ^{14}C DOPA preparation, giving total blank value of about 50 cpm, including background. One μg of dopamine (free base) formed from this batch of ^{14}C-DOPA corresponded to about 12000 cpm. This value was uncorrected for the self-absorption produced by inorganic salts, originally present in the eluate from the ion exchange column and contaminating the acetone extract. The self-absorption was constant in all experiments (*cf* tables 1 and 2), and the results obtained were therefore directly comparable without correction. The identity of the radioactive compounds present in the eluate was usually established by simultaneous paper chromatographic analyses of some samples by a radiometric technique (fig. 1). The eluate containing the amine was evaporated to dryness under reduced pressure at room temperature, treatment that leaves the catechol amines intact. The dry residue was extracted twice with 2 ml of acidified acetone. After evaporation almost to dryness at room temperature, the acetone extract was spotted on Whatman No 1 filter paper. Separation was performed by three different solvents 1) n-butanol saturated with

N hydrochloric acid (BERTLER *et al.* 1958) 2) methanol-butanol-benzene-water (2:1:1:1) (SENOH & WITKOP 1959) 3) phenol-water (500 g of phenol dissolved in 125 ml water) (cf. SMITH 1958). The distribution of radioactivity on the paper chromatograms was determined as described in detail below. The carrier and the reference compounds were detected by means of the Pauly reagent (cf. SMITH 1958).

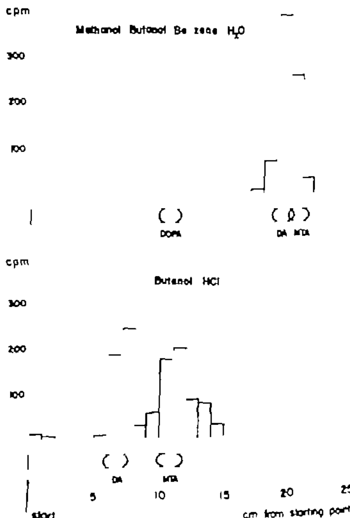


Fig. 1. Radio paper chromatography of extracts from 8 day chick embryo tissue incubated with ^{14}C-DOPA (ENEMAR, FALCK & HÅKANSON 1965) and purified by ion exchange (see text). The solvent systems used are indicated in the figure, as are the positions of the reference compounds. Separation was performed over-night at room temperature.

Dihydroxyphenylalanine	DOPA
Dopamine	DA
3-Methoxytyramine	MTA

Methanol-butanol-benzene-water (2:1:1:1).
Butanol-HCl: n-butanol saturated with N hydrochloric acid.

Measurement of histidine decarboxylase activity

The sample was incubated for one hour with 2 μg ^{14}C-histidine in a total volume of one ml. I some experiments aminoguanidine (histaminase inhibitor) was added to a final concentration of 10^{-4}M After the incubation, trichloroacetic acid was dded to give a final concentration f 5% and a t tal volume of 3 ml. Non-radioactive histamine dihydrochloride, 20–40 μg, was added as carrier After homogenization in the Ultra Turrax homogenizer the precipitate was spun down and discarded. The supernatant was washed twice with four volumes of diethyl ether to remove the trichloroacetic acid. The samples were transferred to centrifuge tubes containing 0.5 ml 5 N N OH and extracted for 5 minutes with 15 ml of n-butan l-chloroform (3 2). After centrifugation at low speed the organic phase was transferred to another tube containing 3 ml 0.03 N sodium hydroxide. After shaking for one minute, the tube was centrifuged, and the organic phase was transferred to another tube containing 3 ml 0.1 N hydrochloric acid and 4 ml heptane. The mixture was shaken for one minute and then centrifuged. This procedure is essentially that described by BURKHALTER (1962). The final hydrochloric acid fraction contained approximately 60% of the original amount of histamine. Recovery was not affected by the presence of tissue up to 100 mg. Since histamine is not separated entirely from contaminating ^{14}C-histidine by organic extraction, further purification f the amine by paper chromatography was found to be necessary The hydrochloric acid extract was evaporated t dryness on steam bath, and the dry residue was taken up in 3 + 3 drops f alkaline ethanol (25 ml bsolute ethanol + few drops of ammonia) and applied as thin line onto Whatman No 1 filter paper strip for chromatography Descending chromatography was usually employed, with solvent system of ethanol-ether-ammonia water (8 10 1 1) (COWGILL 1955) for bout two hours, allowing the solvent front t travel about 20 cm. In some experiments the

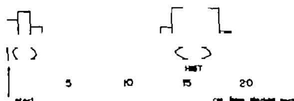


Fig. 2. The distribution of radioactivity on paper chromatogram of an extract from rat bone marrow (HÅKANSON 1964) incubated with 10 μg ^{14}C-histidine (HID) for one hour and treated for measurement of histidine decarboxylase activity Ascending chromat graphy was performed over-night in solvent system f ethanol-ether-water-ammonia (8 10 2 1). With this technique the Rf-value of histamine (HIST) is 0.55, and the corresponding figure for 1,4-methylhistamine is 0.60 The radioactive impurity of unknown identity present in the partly purified preparation of ^{14}C-histidine used (see Methods) was found to have Rf value of 0.40. In this experiment the radioactivity of this compound produced peak (not detectable on this scale) corresponding t a total of bout 100 cpm.

Identity of the metabolite formed was tested by ascending chromatography overnight in two other solvent systems n-butanol saturated with N hydrochloric acid or n-butanol-pyridine-water (1 1 1). The chromatogram was dried at 65 The dried paper was dipped in a solution of 0.25% ninhydrin in acetone, and the ninhydrin-positive spots were developed by heating at 100 for a few minutes. In some experiments (fig. 2) the amount of ^{14}C histamine formed was determined by passing the paper strip in 0.5–1 cm portions under the window f a Tracerlab gas flow-counter (with a background value of about 25 cpm), equipped with a mechanical device for the automatic scanning of paper chromatograms. A lead shield prevented interference of the remaining part of the strip with the radioactivity of the area being counted. At least 1000 counts were taken for each portion. Quantitative results were obtained by integrating the counts for the histamine-containing area and subtracting the background. One μg of histamine formed from this batch of ^{14}C-histidine corresponded to about 1000 cpm. No difference was observed between the values obtained before and after ninhydrin treatment. For the more quantitative estimation, however, the histamine-containing part of the ninhydrin-treated paper chromatogram was cut and eluted overnight with 5 ml of ethanol containing few drops of ammonia per 25 ml. The ethanol fraction was evaporated to dryness on planchettes for measurement of radioactivity in the Frieseke-Hoepfner gas flow-counter By this method, each μg of histamine (free base) formed corresponded to about 30 000 cpm, without correction for self-absorption. Under the conditions used the self-absorption of the samples was constant. The blank value did not exceed the background. All values given below refer to the ethanol-eluted samples.

Measurement of glutamic acid decarboxylase activity

The sample was incubated with 2 μg ^{14}C-glutamic acid in the presence of 40 μg non-radioactive γ-amino-n-butyric acid (GABA) in a total volume of one ml for one hour Incubation was interrupted by the addition of trichloroacetic acid to final concentration of 5%. After homogenization the precipitate was spun down and discarded. The supernatant was washed twice with four volumes of diethyl ether to remove the trichloroacetic acid. After this treatment the pH of the samples varied between 3 and 4. Deviations from these values were corrected by the addition of small amounts of 0.1 M acetate-buffer pH 3.5. The sample was then transferred to the Dowex 50 column, previously treated with 10 ml M ammonium acetate-acetic acid buffer pH 3.5 and 5 ml redistilled water All contaminating glutamic acid was eliminated from the column by washing with 10 ml 0 4 M acetate buffer, pH 3.5 and 30 ml redistilled water GABA could then be eluted quantitatively with 10 ml 0.005 N mmonia in 65% ethanol. The ethanol fraction was evaporated to dryness on steam bath. The dry residue was extracted twice with 2 ml acetone containing 0.1 ml of 2 N hydrochloric acid per 100 ml, and the cetone extract was evaporated to dryness on planchettes for measurement of radioactivity Under the conditions used the self-absorption of the samples was constant. Without correction for the self-absorption, one μg GABA formed from our batch of ^{14}C-glutamic acid corresponded to about 60,000 cpm. The blank value did not exceed the background value. The procedure adopted for the ion exchange separation was found to elute at least one other metabolite of glutamic acid together with GABA. Though only traces of added glutamine occurred i the eluate, added α-amino-n-butyric acid (AABA) was recovered quantitatively in the GABA-containing fraction. It was essential to make radiometric paper chromatographic analysis of at least one sample in a series, because of the possible presence of AABA or other metabolites of glutamic acid in the eluates of tissue extracts incubated with ^{14}C-glutamic acid. In these studies the final acetone extract was reduced to small volume by evaporation (at room temperature) and streaked on to Whatman no 1 filter paper Descending chromatography was generally utilized, with a solvent system of methanol-acetic acid (100 1) or ethanol-ammonia-water

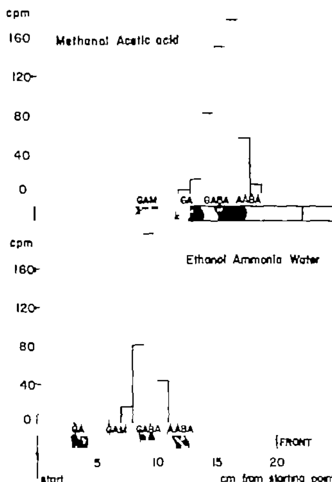


Fig. 3. Radio paper chromatography of extracts from rat brain incubated with ^{14}C-glutamic acid. One g of the tissue was homogenized in 10 ml ice cold 0.1 M phosphate buffer pH 7.2, and centrifuged at 20 000 g for 10 min. One ml of the supernatant was incubated at pH 7.2 with 2 µg ^{14}C-glutamic acid in the presence of 40 µg GABA in nitrogen atmosphere at 37° After incubation for one hour the samples were purified (see text). The metabolites were separated by descending paper chromatography for about two hours. The solvent systems used are indicated in the figure, as are the positions of the reference compounds.

GA *Glutamic acid.*
GAM *Glutamine.*
GABA γ-amino-n-butyric acid.
AABA α-amino-n-butyric acid.
Methanol-acetic acid (100 1).
Ethanol-ammonia-water (10 1 1).

(10 1 1) for about two hours, allowing the solvent fro t to travel about 20 cm (fig. 3). In some experiments separation was performed in phenol-water or phenol-ammonia-water (cf. SMITH 1958). The chromatogram was dried t 65 and developed by ninhydrin and subsequent heating. The distribution of radioactivity along the paper chromatogram was studied by automatic scanning with the Tracerlab gas flow-counter

Results and Discussion

The number of cpm corresponding to a certain amount of amine produced during incubation with the labelled precursor was established by experiments in which known amounts of ^{14}C-labelled amines were added to tissue samples (table 1) The variation was found to be small.

The reliability of the methods was tested by model experiments with and without tissue (table 2) and by experiments involving use of internal standards (table 3)

Enzyme activity was found to vary linearly with the amount of tissue extract and the duration of the incubation (fig. 4)

All values given in the tables and in the figs. are uncorrected the consistency of the results obtained under the standard conditions made correction for self-absorption unnecessary

The paper chromatographic methods for identification of the metabolites formed were chosen for their ability to separate the amines from their respective precursors (SENOH & WITKOP 1959 COWGILL 1955) and metabolites (BERTLER *et al* 1958 SENOH & WITKOP 1959 LINDAHL 1960 a & b) (fig. 1 and 3).

In studies of DOPA decarboxylase activity with tissue or crude tissue extracts the formation of radioactive compounds other than the expected product has been observed (fig. 1) At present it seems probable that these compounds are O-methylated derivatives of the primary amine (AXELROD,

Table 1

Recovery in counts per minut of ^{14}C-labelled amines added to tissue samples of the same weight.

Compounds	Amount added µg (free base)	Specific activity mc/mM	Radioactivity recorded in cpm Mean ± S.E.M. (number of samples)
Dopamine	0.1	5.75	1153 ± 33 (12)
Histamine	0.1	21.3	2800 ± 58 (24)
GABA.	0.1	3 72	1510 ± 46 (12)

The ^{14}C-labelled amine was added together with carrier to 50 mg minced rat liver suspended in 1 ml 0.1 M phosphate buffer pH 7 0. Except for the incubation, the samples ere treated by the routine procedures.

Table 2

Recovery of [14]C-labelled amines added to tissue samples of various weights.

Wet weight of tissue in mg	Radioactivity registered in cpm		
	Dopamine	Histamine	GABA
0	1165	2770	1790
10	1146	2690	1513
20	1072	2860	1400
30	1114	2910	1546
40	1161	3160	1639
60	1707	3060	1595
80	1107	3100	1487
100	1191	2830	1481

[14]C-labelled amines in amounts of 0.1 μg (free base) were added together with carrier to separate samples of minced rat liver suspended in 1 ml 0.1 m phosphate buffer pH 7.0. Except for the incubation, the samples were treated by the routine procedures.

Table 3

Recovery of standards and internal standards.
Experiments on glutamic acid decarboxylase from rat brain.

Description of samples and standards	Radioactivity registered in cpm
0.1 ml extract	1990
0.1 ml extract	1700
0.1 ml extract	2086
0.1 μg [14]C-GABA	1290
0.1 μg [14]C-GABA	1495
0.1 μg [14]C-GABA	1325
0.1 ml extract + 0.1 μg [14]C-GABA	3200
0.1 ml extract + 0.1 μg [14]C-GABA	3200
0.1 ml extract + 0.1 μg [14]C-GABA	3340

Rat brain extracts was prepared as described elsewhere (fig. 3). The extract was incubated with [14]C-labelled glutamic acid by the routine procedure. Standards and internal standards were run parallel with the samples (see text).

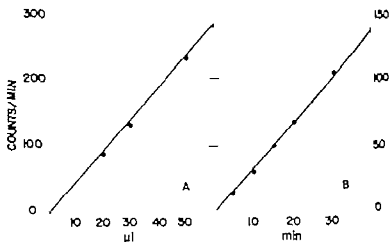


Fig. 4. The effect of enzyme concentration (A) and incubation time (B) on DOPA decarboxylase activity. The enzyme was obtained by homogenizing rat stomachs, wet weight 20 g in ice-cold 0.1 M phosphate buffer pH 7.6, and precipitating with ammonium sulphate. The fraction precipitated at between 40 and 50 % saturation was taken up in 10 ml buffer and dialysed over-night. The incubation was performed in a volume of one ml at pH 7.0 with 3 µg ^{14}C-DL-DOPA and 1 µg pyridoxal-5-phosphate. In A the incubation time was one hour. In B the amount of extract added was 50 µl.

ALBERS & CLEMENTE 1959, AXELROD & TOMCHICK 1958). The possible presence of such methylated metabolites thus make it preferable to give the results in cpm of decarboxylated derivatives rather than in micrograms of primary amine produced. A similar situation exists with histamine and its metabolite 1,4-methylhistamine, since in many species N (ring)-methylation is the main pathway for histamine catabolism (SCHAYER 1959). The N-methylated metabolite of histamine is extracted together with histamine by the butanol-chloroform mixture used and separates poorly from histamine with the solvent system routinely employed for the paper chromatographic isolation of this amine. However, separation is easily obtained with other solvent systems (LINDAHL 1960a).

POTTER (1944) claimed that an enzyme assay, to be valid, must satisfy three conditions: activity must be proportional to the amount of enzyme added (enzyme extract or tissue sample), activity must be constant during the incubation period, and the assay must be carried out at saturating substrate concentration. Usually the third condition is difficult to fulfil with expensive labelled substrates. This restriction is applied more for practical than for theoretical reasons. In principle an enzyme may be assayed at any substrate concentration provided conditions are identical for all samples and provided the reaction rate is linear with time. It is evident, however, that the employment of low "physiological" amounts

of substrate may introduce serious errors in the determination of high enzyme activities. This was clearly illustrated in studies on rat kidney cortex, a rich source of DOPA decarboxylase (ROSENGREN 1960), in which the standard amount of ^{14}C DOPA was quantitatively transformed to dopamine in less than one hour. Consequently in this experiment the enzyme activity recorded gave no adequate information about the enzyme concentration. To obtain a linear relationship between enzyme activity and enzyme concentration the incubation time was shortened and the substrate concentration increased. The results and conditions of these experiments are given in fig. 5. If more than 10–20 % of the substrate is consumed in the reaction, it is recommended that the experimental conditions be adjusted along the lines indicated above. It should be emphasized that radiometric methods for the measurement of amino acid decarboxylase activity although useful for some purposes, must be applied with caution.

The estimate of enzyme activity may be modified by several factors. Thus, the results obtained may be affected by 1) cell membrane pheno-

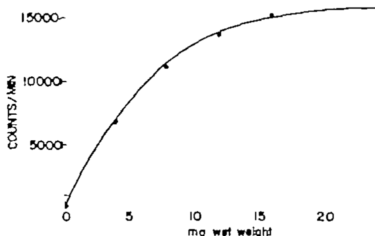


Fig. 5. DOPA decarboxylase activity of minced rabbit kidney cortex plotted against the wet weight of the samples. The figure shows the results of two experiments, identical except for the amount of substrate added. Each point represents the mean value of two determinations. The curves have been fitted visually.

○———○ 3 µg ^{14}C DL DOPA

●———● 3 µg ^{14}C DL DOPA + 300 µg non-radioactive DL DOPA.

All incubations were performed in one ml 0.1 M phosphate buffer pH 7.0, in nitrogen atmosphere at 37° for 20 min.

mena, 2) the presence of endogenous substrate, 3) the concentration of co-factors and 4) the activities of other enzymes involved in the metabolism of the substrates or the products.

1) Some information on the influence of cellular permeability may be obtained by simultaneous study of minced and homogenized tissue, assuming that mincing leaves the majority of the cells intact whereas homogenization causes disruption of the cells. Active or blocked intracellular uptake of the substrate may profoundly affect the results of the assay of enzyme activity in tissue slices or in suspensions of intact cells. Evidence indicating the importance of such mechanisms in amine metabolism has been presented (DAY & GREEN 1962).

2) The presence of endogenous substrate may serve to alter the specific radioactivity of the added substrate, and a misleading result may consequently be obtained. Such sources of error may be eliminated by using smaller amounts of tissue, thereby diminishing the amount of interfering substrate, or alternatively by increasing the amount of substrate added. Another possible way to solve this problem would be to ascertain the amount of endogenous substrate present in the sample and take this into account when calculating the results. Similar problems have been discussed by REINER (1959) Since DOPA and 5-hydroxytryptophan are believed to be further metabolized immediately after biosynthesis *in vivo*, the problem does not seem to arise with these substrates. The enzyme that decarboxylates DOPA and 5 HTP however is believed to attack several aromatic amino acids (ROSENGREN 1960 LOVENBERG, WEISSBACH & UDENFRIEND 1962), indicating that normally occurring amino acids of aromatic nature may act as competitive inhibitors in the determination of DOPA decarboxylase activity

3) The only known cofactor of amino acid decarboxylases is pyridoxal-5-phosphate which is indispensable for enzyme activity Non-dialysed enzyme material contains varying amounts of the co-factor usually the enzyme activity is enhanced by the addition of pyridoxal-5-phosphate, probably indicating that the cofactor is loosely bound and dissociates from the enzyme in the incubation medium. Small amounts of pyridoxal-5-phosphate must be added for maximal velocity (HÅKANSON 1963 HÅKANSON, unpublished observations)

4) Besides the decarboxylases there are *other enzymes* involved in the anaerobic metabolism of amino acids. The activities of these enzymes will diminish the amount of added ^{14}C-labelled substrate and thereby affect the results. Glutamic acid decarboxylase has been studied by measuring the $^{14}CO_2$ produced from glutamic acid 1 ^{14}C (ALBERS, KOVAL, MCKHANN & RICKS 1961). However $^{14}CO_2$ is produced enzymatically

from this substrate not only by glutamic acid decarboxylase but also via transamination and subsequent oxidation. Although this source of error may be eliminated to a large extent by use of anaerobic conditions or inhibitors (ALBERS *et al* 1961), determining the amount of GABA produced seems to be a more accurate way of studying this enzyme However further metabolism of the amines produced is evidently also a source of error Since incubation is performed in a nitrogen atmosphere, the activities of monoamine and diamine oxidase should be of minor importance. N and O-methylation, acetylation, transamination (GABA) and possibly conjugation would seem to be of greater consequence. The use of inhibitors (AXELROD & LAROCHE 1959 PLETSCHER, GEY & ZELLER 1960 ZELLER 1963 SOURKES & D'IORIO 1963) has proved to be of great value in diminishing the interference by some of these mechanisms (HÅKANSON & MÖLLER 1963 HÅKANSON, unpublished observations) It should be noted that some inhibitors of monoamine oxidase and catechol-O-methyl transferase, for example, interfere with the activity of amino acid decarboxylases as well (cf CLARK 1963 HÅKANSON & OWMAN 1965). In some experiments incubation in the presence of carrier was found to inhibit the further metabolism of labelled amines produced, without noticeably affecting the amino acid decarboxylase activity (HÅKANSON, unpublished observations). Finally the presence of other agents, e.g. some metal cations, may affect the enzyme activity by reacting with the coenzyme pyridoxal 5-phosphate (CLARK 1963)

Results obtained by these and similar methods give only an *estimate* of the enzyme concentration in a tissue or tissue extract. The special conditions of each experiment must be considered in the final interpretation

Summary

Rapid, simple and sensitive methods for *in vitro* study of some amino acid decarboxylases are described. After incubating tissue samples with the ^{14}C-labelled amino acid, the amine formed is isolated by ion exchange chromatography solvent extraction or paper chromatography (or both) for subsequent determination of radioactivity The use of ^{14}C-labelled substrates permits the estimation of amino acid decarboxylase activity in samples containing low amounts of enzyme, such as isolated cells or small pieces of tissue.

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From the Department of Biochemistry (Professor S. Darling, Ph.D.),
the Royal Dental College, Aarhus, Denmark

Crystallization and Preliminary Characterization of a Dicoumarol Metabolite in the Faeces of Dicoumarol-Treated Rats

By

Flemming Christensen

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It has been shown that ingestion of (methylene-^{14}C)-dicoumarol by rats leads to the excretion of several radioactive metabolites in both faeces and urine, the former being the more important (CHRISTENSEN 1965). No attempt was made to identify the individual metabolites, but several of them were characterized by their paper chromatographic behaviour (R_F-values in 3 different solvent systems) and their colour reactions with certain diazotized aromatic amines.

In this paper the isolation and crystallization of small amounts of the most prominent of these metabolites from the faeces and urine of dicoumarol-treated rats are described. Although a final identification of the metabolite, referred to as B-055 has not yet been achieved, some preliminary investigations reported here may indicate part of its structure.

Hitherto practically nothing has been known about the chemical fate of dicoumarol in mammalia (see WILLIAMS 1959) although some information is available as to the chemical fate of coumarin itself various hydroxy coumarins and the derived dicoumarol known as tromexan ® (ethyl biscumacetas (NFN)) (see also WILLIAMS 1959). Knowledge about the chemical transformations of the latter compounds might be taken to indicate that reactions involving hydroxylations or conjugation with either glucuronic acid or sulphuric acid, or both, should also be considered for dicoumarol. Further as methoxylation is among the known detoxifying reactions for phenolic compounds in mammalia (see WILLIAMS 1959), this possibility was also considered Lastly a splitting of dicoumarol (e.g. into its constituent moieties) is theoretically possible.

As it seemed likely that the metabolite B-055 could derive from di-

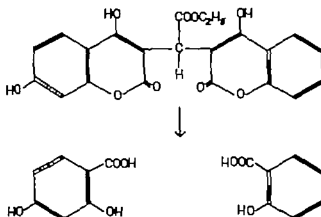


Fig. 1 Reaction of 7-hydroxytromexan with potassium hydroxide, showing the formation of salicylic acid and 2,4-dihydroxybenzoic acid.

coumarol by one of the routes mentioned, it was submitted to various degradation reactions and also compared with some known hydroxy and methoxy derivatives of dicoumarol and 4-hydroxycoumarin.

Because of the small amount available for chemical examination this was limited to

1 Treatment with diluted strong acid, to hydrolyse a glucuronide or sulphuric ester 2. Incubation with β-glucuronidase, to split a glucuronide 3 Test for methoxy groups. 4 Degradation with potassium hydroxide. This last reaction, which has been useful in determining the structure of dicoumarol (STAHMANN *et al* 1941) and 7-hydroxytromexan (BURNS *et al* 1953), is based on the principle that coumarins without hydroxyl groups in the aromatic ring are degraded to salicylic acid, whereas coumarins possessing one such group in the aromatic ring are degraded to a dihydroxybenzoic acid. The degradation of 7-hydroxytromexan to salicylic acid and 2,4-dihydroxybenzoic acid may thus be represented by the equation shown in fig. 1 Derivatives of dicoumarol are probably degraded in a similar way and valuable information about an unknown compound of this kind (e g. B-055) should be obtained from knowledge of the reaction products formed This does seem to apply to metabolite B-055 as will be shown later

Experimental

1. Isolation, purification and crystallization of metabolite B-055

Fifty rats were each injected intravenously with about 5 mg of dicoumarol, and faeces and urine were collected separately for the succeeding 24 hours.

The dried and finely grounded faeces (34 g) were shaken mechanically for 4 hours with 1 litre of acetone M HCl (90 10, v/v) and the suspension was then filtered. The filtrate, concentrated under reduced pressure to 100 ml and saturated with solid sodium chloride, was extracted 3 times with 200 ml of chloroform. The combined extracts were extracted twice with 50 ml of 2.5 M NaOH, and the extract after cooling in ice water was acidified with conc. HCl. The acid solution was saturated with sodium chloride, and extracted 3 times with 200 ml of chloroform. The combined chloroform extracts were then dried under reduced pressure. The resulting brownish material was dissolved in aqueous ammonia (3 M), and applied to several (16) Whatman 3 MM filter papers, each sheet being loaded with as much material as possible. After development by butanol 3 M-aqueous ammonia (1 1 /) the position of the metabolite on the chromatograms was localized by spraying a strip of each paper with diazotized o-dianisidine. The zone containing the metabolite was cut from each chromatogram and eluted with 3 M-aqueous ammonia. The eluates ere combined, pplied to a new series of filter papers and chromatographed again, as described above. The eluates from these chromatograms were then rechromatographed with ethyl acetate 3 M-aqueous ammonia (1 1 v/v). The metabolite was finally eluted ith 3 M-aqueous ammonia and the eluate evaporated to dryness. The slightly brownish remnant was treated with acetone (20 ml), but some material remained undissolved. This was removed by centrifugation and discarded, as it did not react with the o-dianisidine colour reaction used for tracing the metabolite. The acetone solution was dried under reduced pressure, and a white material resulted. It was dissolved in small volume of boiling cyclohexanone on cooling a yield of about 2 mg of colourless prisms was obtained.

A small amount of the metabolite was also isolated from the combined urine specimens of the dicoumarol-treated rats. The urine, acidified with conc. HCl and saturated with solid sodium chloride, was extracted 3 times with 2 vol. of ethyl acetate. The combined extracts were dried under reduced pressure, and a brown residue was obtained. From this the metabolite was isolated in a pure form by the same methods as those used for faeces. About 1 mg of colourless prisms was obtained by the final recrystallization from cyclohexanone.

The crystallin material isolated from both faeces and urine were, judged by paper chromatography and paper electrophoresis, identical and pure. Both of the compounds dissolved in 2.5 M N OH showed peak bsorption of light at 315 mμ and a minimum at 265 mμ. Mp > 350°

The crystalline material is referred to as B-055 in this paper

2. *Analytical methods*

Ultraviolet spect were obtained by means of a Zeiss PMQ-II spectrophotometer All the compounds examined were dissolved in 2.5 M NaOH

Paper chromatography was carried out as described by CHRISTENSEN (1964). The one-dimensional technique was used, and the chromatograms were developed by one of 3 solvent systems

Solvent system 1 n-butanol 3 M-aqueous ammonia (1 1 /v)
Solvent ystem 2 benzene acetic acid water (2 2 1 /v)
Solvent system 3 ethyl acetate 3 M-aqueous ammonia (1 1 /v)

Detection of coumarin compounds on the paper chromatograms was carried out by the colour reactions with diazotized o-dianisidine and diazotized sulphanilic acid, as described by CHRISTENSEN (1964).

Detection f hydroxybenzoic acid on paper chromatograms was based partly on the colours of the fluorescent spots when the chromatograms were viewed in the light of an

ultraviolet lamp (max. energy at 360 mμ) and partly on the colours obtained after spraying the papers with diazotized sulphanilic acid as described above. The coupling, however, was performed at pH 9 instead of at pH 7.

The paper electrophoretic mobilities of some dicoumarol derivatives were also studied, the experiments being carried out with a closed strip electrophoresis apparatus (LKB). The sample was applied to Whatman no. 1 filter paper strips, and M/20 borate buffer pH 9.0 was used in the electrode vessels. The movements at room temperature of the individual spots were determined after 5 hours with 300 volts, the spots being detected on the papers by the o-dianisidine colour reaction mentioned above.

Acid treatment of metabolite B-055. In one experiment a small sample of the metabolite was boiled with 3 M HCl for 4 hours. In another experiment a small sample of it was kept at 100° with 8 M HCl in a sealed tube for 24 hours. Both acid solutions were extracted twice with 3 vol. of ethyl acetate, and the combined extracts from each were dried under reduced pressure. The residues, dissolved in a small volume of chloroform, were applied to two one-dimensional paper chromatograms and developed by solvent system 1.

Incubation with β-glucuronidase. Small amounts of metabolite B-055 were incubated for various times (3–18 hours) with β-glucuronidase (Sigma) in phosphate buffer pH 6.8. After this treatment the reaction mixture was acidified and extracted with ethyl acetate. The procedure was then the same as described under acid treatment.

Glucuronic acid was determined by the naphthoresorcinol method as modified by PAUL (1951) and cited by MEAD *et al.* (1958 a).

Degradation of coumarin compounds with potassium hydroxide. In a platinum crucible, 100–500 μg of the compound to be examined was thoroughly mixed with 500 mg of finely powdered KOH. The mixture was kept at 300° for 10 min, cooled and dissolved in water. The clear solution obtained was transferred to a glass-stoppered test tube and acidified with conc. HCl, and the volume was made up to 5 ml with water. The acid solution was then extracted twice with ethyl acetate (2 vol.), and the combined extracts were dried under reduced pressure. The residue was dissolved in a small volume of chloroform and known fractions of the resulting solution were applied to each of two paper strips for one-dimensional paper chromatography in system 1 and 2. Standards of salicylic acid and 2,3-, 2,4-, 2,5- and 2,6-dihydroxybenzoic acids were also applied to the papers. After development the spots were traced on the chromatograms by the methods already described. The properties (R_F-values, fluorescence, colour reactions) of the degradation products and of the various known benzoic acid derivatives were noted, and by means of these the unknown compounds were eventually identified.

Test for methoxy groups. This test was carried out as described by FEIGL (1956) on microgram quantities of metabolite B-055 and of some known methoxycoumarin derivatives.

3. *Reference compounds*

The various *dihydroxybenzoic acids* (2,3-, 2,4-, 2,5- and 2,6-) used were all purchased from Light & Co. Ltd., salicylic acid was obtained from B.D.H.

4-hydroxycoumarin (purum) was purchased from Fluka.

4,7-dihydroxycoumarin was prepared by the method of SONN (1917). The compound was recrystallized from hot water.

4-hydroxy-7-methoxycoumarin was prepared as described by SONN (1917) by treating the above-mentioned compound with dimethyl sulphate. It was recrystallized from hot 50% (w/v) ethanol.

Dicoumarol (=3,3′-methylene bis-(4-hydroxycoumarin)). A commercial preparation was used.

7,7'-dihydroxydicoumarol (=3,3' methylene-bis-(4,7-dihydroxycoumarin)) was prepared as described by AMIARD & ALLAIS (1947)

*7-hydroxydicoumarol**) and *7-methoxydicoumarol**) were obtained as gifts. (See acknowledgements).

7,7'-dimethoxydicoumarol (=3,3' -methylene-bis-(4-hydroxy-7-methoxycoumarin)) was prepared by condensing 4-hydroxy-7-methoxycoumarin (25 mg in 25 ml of boiling water) and 0.1 ml of formaldehyde (35 % w/w) The reaction mixture was left for 48 hours at room temperature, and the precipitate was filtered off, washed and dried. It was recrystallized from boiling cyclohexanone, from which colourless prisms separated on cooling.

Results

The R_f-values and colour reactions of metabolite B-055 and the various reference compounds studied are shown in table 1 It is evident from these that B-055 cannot be identical with any of the compounds. It should be mentioned that the metabolite isolated from faeces was, judged by its R_f-values and colour reactions, identical with that isolated from urine and with the radioactive compound B-055 previously detected in the faeces of (methylene [14]C)-dicoumarol treated rats (CHRISTENSEN 1965).

Table 1

R_f-values and colour reactions of metabolite B-055 and some derivatives f dicoumarol and 4-hydroxycoumarin.

Compounds	R_f-values *)			Colours	
	syst. 1	syst. 2	syst. 3	diaz. o-dianisid *)	diaz. sulph. acid)
metabolite B-055	0.55	0.82	0.11	purple	yellow
dicoumarol	0.78	0.94	0.31	purple	yellow
7-hydroxydicoumarol	0.33	0.81	0.01	violet	yellowish-brown
7 7 -dihydroxydicoumarol	0.01	0.01	0	violet	yellowish-brown
7-methoxydicoumarol	0.79	0.94	0.42	violet	yellow
7 7 -dimethoxydicoumarol	0.79	0.95 (t)	0.42	violet	yellow
4-hydroxycoumarin	0.53	0.32	0	violet	yellow
4,7-dihydroxycoumarin	0.04	0.02	0	violet	orange
7-methoxy-4-hydroxy coumarin	0.46	0.29	0	violet	yellow

) Solvent system 1 n-butanol 3 M-aqueous ammonia (1 1 /v).
Solvent system 2 benzene acetic acid water (2:2 1 /v).
Solvent system 3 ethyl acetate 3 M-aqueous ammonia (1 1 /v).

(t) means tailing.

) Coupling performed t pH 7

) Unsymmetrically substituted dicoumarol derivatives are named as proposed by ABRAMOVITCH & GEAR (1958).

Table 2

Electrophoretic separation of metabolite B-055, dicoumarol, 7 hydroxydicoumarol and 7 methoxydicoumarol on Whatman N 1 papers, with M/20 borate buffer pH 9 0 at 300 volts for 5 hours.

Compounds	Movement cm
metabolite B-055	9.5
dicoumarol	11 6
7-hydroxydicoumarol	9.9
7-methoxydicoumarol	8 7

The electrophoretic mobilities of metabolite B-055 and some of the reference compounds are shown in table 2. It should be added that only one zone reacting with diazotized o-dianisidine was detected on electropherograms of crystalline B-055

Results for the ultraviolet spectra of metabolite B-055 and some di coumarol and 4-hydroxycoumarin derivatives are summarized in table 3 It appears that the compound B-055 exhibits maximum light absorption at wavelength 315 mμ, which is close to that for dicoumarol Its absorption minimum is, however shifted towards a slightly longer wavelength (265 mμ) compared with that of dicoumarol (260 mμ) The ultraviolet spectrum of B-055 seems to agree more with that of a dicoumarol deriva

Table 3

Characteristics of the ultraviolet spectra f metabolite B-055 and some derivatives f dicoumarol and 4-hydroxycoumarin. The wavelengths of the bsorption maxima (λ_{max}) and minima (λ_{min}) are shown for the various compounds in 2.5 M N OH)

Compounds	Wavelength mμ	
	λ_{max}	λ_{min}
metabolite B-055	315	265
dicoumarol	314	260
7-hydroxydicoumarol	327	274
7 7′-dihydroxydicoumarol	257 336	246, 278
7-methoxydicoumarol	316	?65
7 7′-dimethoxydicoumarol	247 316	?67
4-hydroxycoumarin	285	250
4 7-dihydroxycoumarin	252, 324	273
4-hydroxy 7-methoxycoumarin	304	266

tive than with that of a simple 4-hydroxycoumarin derivative. The effects of introducing hydroxyl or methoxyl groups into dicoumarol and 4-hydroxycoumarin on the absorption maxima and minima will also be apparent from the table.

The acid treatment of metabolite B-055 did not cause any recognizable alteration either in its chromatographic behaviour or in its staining reactions with diazotized sulphanilic acid and diazotized o-dianisidine, compared with an untreated control sample. Glucuronic acid was not liberated during the acid treatment.

The incubation of metabolite B-055 with β-glucuronidase caused alterations neither in its chromatographic behaviour nor in its staining reactions with diazotized aromatic amines compared with those of the untreated control compound. No glucuronic acid was released during the incubation.

Tests for methoxy groups were carried out on metabolite B-055 and on some of the reference methoxycoumarins. The colours obtained with the chromotropic acid used differed somewhat from substance to substance. It may be noted that no colour was obtained with dicoumarol, whereas B-055 gave a distinct bluish colour reaction.

Degradation of coumarin compounds with potassium hydroxide. In order to examine the reliability of this test various coumarin compounds were degraded with KOH as described, and the reaction products were identified by the methods already mentioned (see also table 4). The products thus obtained (table 5) all agreed with those to be expected.

Table 4

R_F-values, ultraviolet fluorescence and colours with diazotized sulphanilic acid for salicylic acid and the various dihydroxybenzoic acids studied. The properties listed were used for the identification of the products obtained by the alkaline degradation of coumarin derivatives as shown in table 5.

Compounds	R_F-values*)		Colours of fluorescence (360 mμ)	Colours with diaz. sulph. acid **)
	syst. 1	syst. 2		
salicylic acid	0.60	0.77	dark blue	yellow
2,3-dihydroxy benzoic acid	0.18 (t)	0.25	blue	yellowish-brown
2,4-dihydroxy benzoic acid	0.09	0.13	no	brown
2,5-dihydroxy benzoic acid	0.11 (t)	0.09	light blue	yellowish-brown
2,6-dihydroxy benzoic acid	0.69	0.06	no	brown

*) Solvent system 1: butanol: 3 M-aqueous ammonia (1:1 v/v).
Solvent system 2: benzene: acetic acid: water (2:2:1 v/v).
**) *Coupling performed at pH 9.*
(t) means tailing.

Table 5

Main products obtained by degrading the various compounds listed with potassium hydroxide at 300° for 10 min. The products were identified as described in the text (conf. also table 4).

Compounds fused with KOH	Main products obtained by fusion with potassium hydroxide
salicylic acid	salicylic acid
2,3-dihydroxy benzoic acid	2,3-dihydroxy benzoic acid
2,4-dihydroxy benzoic acid	2,4-dihydroxy benzoic acid
2,5-dihydroxy benzoic acid	2,5-dihydroxy benzoic acid
2,6-dihydroxy benzoic acid	2,6-dihydroxy benzoic acid
4-hydroxycoumarin	salicylic acid
4 7-dihydroxycoumarin	2,4-dihydroxy benzoic acid
7-methoxy-4-hydroxycoumarin	2,4-dihydroxy benzoic acid
dicoumarol	salicylic acid
7-hydroxydicoumarol	salicylic acid + 2,4-dihydroxy benzoic acid
7-methoxydicoumarol	salicylic acid + 2,4-dihydroxy benzoic acid
7 7'-dimethoxydicoumarol	2,4-dihydroxy benzoic acid
7 7'-dihydroxydicoumarol	2,4-dihydroxy benzoic acid
metabolite B-055	salicylic acid + 2,4-dihydroxy benzoic acid

On degradation of B-055 with potassium hydroxide, salicylic acid and 2,4-dihydroxy benzoic acid were identified as the reaction products. The yields, on a molar basis, of these two compounds, judged visually from the colour intensities of the spots on the chromatograms, differed somewhat, the yield of salicylic acid being higher than that of 2,4-dihydroxy benzoic acid. However it should be noted that approximately the same difference in the yield of reaction products was obtained when samples of salicylic acid and 2,4-dihydroxybenzoic acid were submitted to the degradation procedure

Discussion

As already mentioned, the amount of the crystalline metabolite B-055 isolated from the faeces was only about 2 mg. However from the results of previous studies (CHRISTENSEN 1965) it was calculated that around 50 mg of metabolite B-055 should be present in the faeces of the rats used. From these figures the recovery was calculated to be only about 4%. This low recovery rate must be ascribed primarily to the loss from the repeated paper chromatographic purifications, but also to incomplete extraction of the compound from the original material.

The results of the paper chromatographic and paper electrophoretic studies reported here indicate that metabolite B-055 was obtained in a pure form. Its ultraviolet spectrum characterizes it as a derivative of dicoumarol rather than of 4-hydroxycoumarin. This seems to be supported also by its identity with the radioactive compound B-055 found in the faeces of rats treated with ^{14}C labelled dicoumarol (CHRISTENSEN 1965), as this means that the carbon atom of the methylene bridge of dicoumarol must be present in the metabolite too. The results of the alkaline degradation of B-055 to be discussed below is likewise in accordance with this view.

As already stated, our knowledge of the fate of dicoumarol derivatives in mammalia is limited. In fact, the only information available is about the compound tromexan ® which according to BURNS *et al.* (1953) is hydroxylated to 7-hydroxytromexan to the extent of some 15% in human subjects. In certain animals, however, de-esterification also occurs, producing tromexan acid. In the absence of further information about the chemical fate of dicoumarol and its derivatives, knowledge about the fates of 4-hydroxycoumarin and coumarin might perhaps be useful in predicting the chemical transformations of dicoumarol in animals. Thus MEAD *et al.* (1958a) have shown that 4-hydroxycoumarin is conjugated with glucuronic acid to the extent of 37% in rabbits but it seems to be too acid to be conjugated with sulphuric acid. Coumarin itself is, according to MEAD *et al.* (1958b), apparently hydroxylated at various positions of the coumarin ring system, producing different hydroxycoumarins, which are conjugated with either glucuronic acid or sulphuric acid before they are excreted.

These facts prompted us to study the possibilities that metabolite B-055 might be derived from dicoumarol by the introduction of a hydroxyl group or by conjugation with either glucuronic acid or sulphuric acid or by both. The extreme stability of metabolite B-055 towards treatment with strong acid, however, seems to disprove the last mentioned possibilities. That it could be a glucuronide was further disproved by its stability to treatment with β-glucuronidase. It seemed therefore more likely that B-055 is a hydroxy derivative of dicoumarol. In order to test this possibility the compound was submitted to degradation with potassium hydroxide. The identification of salicylic acid and 2,4-dihydroxy benzoic acid as reaction products strongly indicates that two different aromatic rings, presumably as parts of two coumarin ring systems, must be present in the metabolite. These results, together with the facts already mentioned, are consistent with the view that metabolite B-055 is identical with 7-hydroxydicoumarol. This assumption was, however, easily disproved by a direct comparison of their properties (R_F-values, electrophoretic mobilities

and ultraviolet spectra) As methoxylation has been described as a means of detoxifying phenolic compounds in animals (see WILLIAMS 1959) the possibility that metabolite B-055 might be a methoxy derivative of 7-hydroxydicoumarol was considered a positive test for methoxy groups strengthened the suspicion. The properties of metabolite B-055 were therefore compared with those of 7-methoxydicoumarol, but again several differences were disclosed and excluded the identity of the two compounds. The properties of B-055 were finally compared with those of some other derivatives of dicoumarol and 4-hydroxycoumarin, but considerable differences were again noted.

Although the small amounts of B-055 available for examination did not permit its definitive identification valuable information about its structure has been obtained. Thus the results presented favour strongly the assumption that B-055 is derived from dicoumarol by introduction of a hydroxyl group at position 7 in one of the two coumarin ring systems. Nevertheless, it must be admitted that it is difficult at present to propose a structure that will account for all available experimental information (e.g. the positive test for methoxy groups) Thus further investigation is essential for solving the problem.

Summary

Small amounts of a dicoumarol metabolite previously detected in the tissues, urine and faeces of dicoumarol treated rats have been isolated from the last two sources, purified and crystallized. The metabolite has been further characterized by its behaviour on paper chromatography and paper electrophoresis, by its colour reactions with diazotized sulphanilic acid and o-dianisidine and by its ultraviolet spectrum.

The metabolite was shown to be stable towards treatment with strong acid and incubation with β-glucuronidase After alkaline degradation, salicylic acid and 2,4-dihydroxy benzoic acid were identified as reaction products. Further a positive test for methoxy groups was obtained.

On the basis of the available experimental information the possible structure of metabolite B-055 is discussed. It is most likely that the compound is a derivative of dicoumarol, which means that both coumarin ring systems and the methylene bridge carbon atom are preserved in the metabolite. The results of the alkaline degradation of B-055 point to the presence of an oxygen atom at position 7 of one of the coumarin ring systems, but the other seems unaltered from that of dicoumarol. The possibility that B-055 is identical with 7-hydroxydicoumarol or 7-methoxydicoumarol was ruled out by direct comparison of their properties.

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From the Research Division AB Pharmacia, Uppsala

Estimation of Anticholinergic Drug Effects in Mice by Antagonism against Pilocarpine-Induced Salivation[1]

By

Wolfgang Richter

(Received November 22, 1965)

Peripheral anticholinergic action may be measured both on isolated smooth muscle preparations stimulated with acetylcholine (MAGNUS 1904) and on whole animals, in which the mydriatic effect of drugs or their antagonism to various effects produced by acetylcholine injection or vagal stimulation are used as endpoints. Vagal effects abolished by anticholinergic drugs are blood pressure fall (KÜHL 1925) intestinal spasm (BROWN & WERNER 1949 HOEKSTRA *et al* 1954 BECKER & MCCARTHY 1960), bradycardia (LENKE 1961 VACHER *et al.* 1965), lachrymation (WINBURY 1949) and salivation (CUSHNY 1920).

Measurement of the inhibitory effect on salivation is one of the standard procedures for estimating anticholinergic activity Among the animal species used for salivation experiments are dogs (CUSHNY 1920), cats (BÜLBRING & DAWES 1945) and rabbits (ISSEKUTZ 1917 CHEN 1954) Only few of the antisalivation methods have been standardized sufficiently for routine use. BROWN & QUINTON (1957) described a standardized and sensitive method with rabbits, based on the antagonism of pilocarpine induced salivation.

The purpose of the work reported here was to discover whether an anti-salivation method could be adapted to mice. After encouraging preliminary experiments, a simple method for routine use has been developed. Details of the technique, results with atropine like reference drugs and some applications of the method to drug screening are described in this paper

[1]) A Short Communication of preliminary results for the mouse salivation method was presented at the Scandinavian Meeting of Pharmacology Lund, 1965.

Materials and Method

Animals Unfasted male albino mice, weighing 22 26 g, from the NMRI strain, were used.

General anaesthesia Urethane, 1.8 g/kg was injected i.p. as a 9% solution in 0 ml/10 g body weight.

Pilocarpine Pilocarpine hydrochloride as a 0.02% solution in 0.9% saline as injected s.c. at a fixed dose of 2.0 mg/kg in 0 1 ml/10 g body weight.

Test compounds Drugs were dissolved in 0.9% saline and were injected s.c. in 0 1 ml/10 g body weight.

Filter paper for collecting saliva Whatman nr 3 MM filter paper available in sheets of 46 × 57 cm, thickness 0.30 mm, was used.

Procedure for measuring salivation inhibiting drug effects For routine screening and measuring drug effects inhibiting salivation, the test compound was injected s.c. one minute after induction of general anaesthesia into two to six animals. The site of injection was always the skin of the neck. Fifteen minutes later, 2.0 mg/kg of pilocarpine were given s.c. under the skin of the back near the tail root, and the mice were placed on their bellies, head downward, on an inclined (9°) aluminium plate covered with filter paper Beginning five minutes after the pilocarpine injection, the animals were moved up the filter paper at intervals of five minutes. The spots of saliva produced on the filter paper were circled with ink for later measurements. Twenty minutes after the pilocarpine injection, the test terminated, and the amount of saliva produced was measured as total spot area per mouse. The area of each spot was estimated by superimposing transparent graph paper and counting the number of 5 × 5 mm enclosed squares, which were used as units of measurement. Fractions of squares were estimated to the nearest tenth.

Fig. 1 shows six mice eleven minutes after pilocarpine injection 0.9% saline had been injected as test compound and the flow of saliva resulted in two rows of spots, each representing collection period of five minutes.

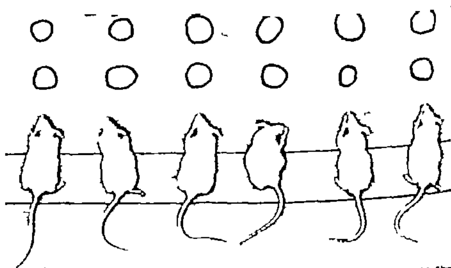


Fig. 1 Six anaesthetized mice, arranged on an inclined aluminium plate covered with filter paper for measuring anti-salivation effect. The test compound as 0.9% saline (control series). Beginning five minutes after injecting 2.0 mg/kg of pilocarpine s.c., the animals were moved up the filter paper t intervals of five minutes. The two rows of saliva spots produced during the first ten minutes of the assay were circled with ink for later measurements.

Methodological Investigations

To study the effect f general anaesthesia on pilocarpine toxicity in mice, the acute toxicity of pilocarpine was determined in 30 unanaesthetized animals and in total of 370 mice injected with both urethane, 1.8 g/kg and pilocarpine doses from 2.0–10.0 mg/kg s.c.

T establish an ptimal dose of pilocarpine, yielding submaximal rate of salivation, the salivary flow after 0.3–5 0 mg/kg of pilocarpine s.c. was measured in five groups of six anaesthetized mice.

The changes in salivary flow rate in five minute periods from 0–60 minutes after sub-maximal standard dose of 2.0 mg/kg of pilocarpine s.c. was investigated in group of 18 male mice.

The magnitude of total salivary flow from 0–20 minutes after a standard dose of 2.0 mg/kg of pilocarpine s.c. was determined in 72 anaesthetized control mice, receiving 0.20 ml of 0.9% saline fifteen minutes before the pilocarpine injection.

On two groups of 12 animals each we looked for any difference in the salivary flow of male and female mice.

The relation between volume of saliva and salivary spot area was studied by applying 0.01–0.30 ml samples (in triplicate) of guinea pig saliva obtained from an anaesthetized animal after injection of 2.0 mg/kg of pilocarpine s.c. to Whatman nr 3 MM filter paper and measuring the area per spot on superimposed transparent graph paper (see fig. 2).

Screening method Three groups of two anaesthetized mice each were injected s.c. with the test compound in doses on the scale (mg/kg) 10.0 5.0, 2.5 Fifteen minutes later pilocarpine was injected s.c. at dose of 2.0 mg/kg, and the salivary flow was determined at 5 minute intervals from 0–20 minutes after pilocarpine injection by determining the mean total salivary spot area per mouse in each group. A mean salivary inhibition of at least 70 0% of the mean salivation from the accumulated controls was considered to indicate salivation inhibitory effect at p = 0.02.

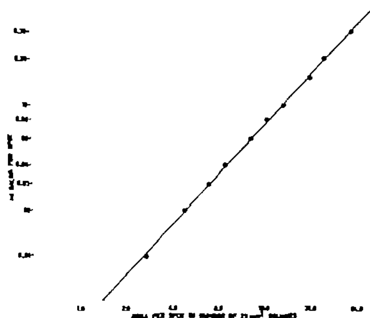


Fig. 2. Graph for converting area of saliva spot into ml saliva.

If the compound exhibited salivation inhibitory effect, lower doses on the same dose scale were tested until no significant inhibitory effect was found at two adjacent dose levels. The lowest dose producing significant salivary inhibition was designated the minimal effective dose.

Method for bio-assay For quantitative determination of the 50% salivation inhibitory dose of an active compound, groups of 4–8 mice were used per dose level, and 3–5 dose levels producing 10–90% inhibition were tested. One or two groups of saline-treated controls were included in each assay The allotment of control animals (C) and animals treated with the various doses of test compound (T) to each of five consecutive groups of six mice was made according to the scheme C, T_1 T_2, T_3, T_4, C. The complete assay of this kind required a total of 30 mice and was performed on one day The time interval between the injection of the test compound and the s.c. injection of pilocarpine was 15 minutes. Salivary flow was determined from 0–20 minutes after the pilocarpine injection. The percentage salivary inhibition was calculated by the formula $100 - \frac{TR \times 100}{C}$, here TR is the average salivary spot area per mouse in the group treated with the test compound and C is the average salivary spot area per mouse of a simultaneously tested control group injected with 0.9% saline. Dose response lines and ED50 values were obtained graphically from a plot of log dose against percentage salivary inhibition. In one duration study the time between test drug and pilocarpine injection was varied, but the salivary flow was always measured from 0–20 minutes after pilocarpine injection.

Methodological Results

From table 1 it can be seen that pilocarpine toxicity is high in anaesthetized mice, the LD50 of subcutaneously injected pilocarpine being about 5 mg/kg. At a dose of 2.0 mg/kg only sporadic deaths occurred within one hour after pilocarpine injection In contrast, the i.p. LD50 in non-anaesthetized mice was found to be 170 mg/kg.

The average salivary flow in ml/mouse from 0–30 minutes after pilocarpine injection is shown in fig. 3 An approximately linear relation between log dose and salivary flow was obtained at doses of 0.3–2.5 mg/kg. At each point the standard deviation did not exceed 25% of the mean.

The salivary flow rate after the standard dose of 2.0 mg/kg of pilocarpine was high and relatively constant for twenty minutes after pilocarpine injection (see fig. 4) The flow then decreased steadily and reached a value corresponding to about 20% of the initial flow at 40–60 minutes after the pilocarpine injection

When the salivary flow 0–20 minutes after 2.0 mg/kg of pilocarpine was compared in male and female mice, it was found, that the males produced about 10% more saliva than the females. The amount of saliva collected corresponded to an area of 45 ± 15 (mean ± stand.dev) squares of 25 mm² for the females, and 52 ± 11 (mean ± stand.dev) squares of 25 mm² for the males. The difference was significant only at a level of P = 0.2.

Table 1

Effect of general anaesthesia on pilocarpine toxicity in mice.

	Dose of pilocarpine chloride in mg/kg s.c.	Number of deaths	Observation time in hours	LD50) mg/kg
Anesthesia)	10.0	8/10	1	
	5.0	6/10	1	5.5 ± 0.7
–	2.0	5/148	1	
No anesthesia.	400.0	20/20	72	
	300.0	19/20	7'	
–	200.0	13/20	72	170 ± 1
–	100.0	2/20	72	

) The LD50 and its standard error were calculated by the method f Miller & Tainter (1944).

) Animals were anaesthetized with 1 8 g/kg of urethane and received pilocarpine injections 15–20 minutes after the onset of anaesthesia.

In a larger series of 72 anaesthetized male control mice, the saliva per mouse collected from 0–20 minutes after the s.c. injection of 2.0 mg/kg of pilocarpine gave a spot area of 56 ± 16 squares of 25 mm² (mean ± stand.dev) per mouse.

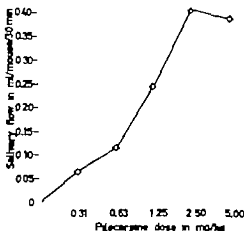


Fig. 3. Relation between pilocarpine dose and salivary flow in mice anaesthetized with 1.8 g/kg of urethane. Each point represents the verage for 5–8 animals.

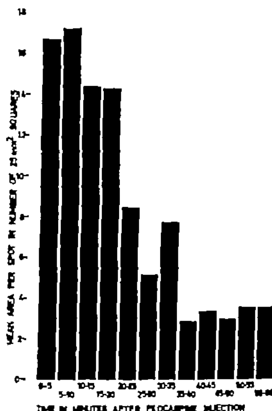


Fig. 4 Salivary flow in anaesthetized mice at 5 minute intervals from 0–60 minutes after a s.c. dose of 2.0 mg/kg of pilocarpine. Each column represents the average for 18 male mice.

This implies that each mouse showing a salivary spot area of 17 squares of 25 mm² or less is to be considered as showing significantly reduced salivation at $p = 0.02$ this figure corresponds to an inhibition of 70 % of the mean salivary flow and is also adopted as the limit for significant effect in the screening method.

Results

Results of screen tests performed on groups of two mice with various doses of a number of atropine-like and other drugs are summarized in table 2. It is seen that hyoscine methonitrate produced the strongest inhibition of salivation, with a minimal effective dose of 10 µg/kg. The minimal effective dose for atropine sulphate in six experiments varied between 10 and 40 µg/kg.

Table 2

Screening of reference drugs for salivation inhibitory activity in mice.

Test compounds were injected s.c. in groups of two unanaesthetized mice. Fifteen minutes later the animals were injected with 2.0 mg/kg of pilocarpine HCl s.c. Salivary flow was determined 0–?0 minutes after the pilocarpine injection. A compound was considered to possess significant salivation inhibitory effect ($0.05 > p > 0.01$) when it produced 70–100 % inhibition at any dose level. From control values obtained for 72 anaesthetized mice treated with saline and pilocarpine, 70 % inhibition corresponds to a saliva spot area of 17 squares of 25 mm² per mouse, i.e. 0.08 ml saliva.

I = Inhibition of salivary flow of at least 70 % — 0 = Inhibition of salivary flow 0–69 %

Test compound	Test no.	Dose in mg/kg 10.0	5.0	2.5	1.25	0.63	0.31	0.16	0.08	0.04	0.02	0.01	0.005	0.002	Minimal effective dose in mg/kg
Atropine sulphate	1	I	I	I	I	I	I	I	I	I	I	0	0	0	0.02
	2				I	I	I	I	I	I	I	0	0	0	0.02
	3				I	I	I	I	I	I	I	I	0	0	0.01
	4				I	I	I	I	I	I	I	I	0	0	0.01
	5				I	I	I	I	I	I	0	0	0		0.04
	6				I	I	I	I	I	I	I	0	0		0.02
Hyoscine methonitrate	1	I	I	I	I	I	I	I	I	I	I	I	0	0	0.01
	2	I	I	I	I	I	I	I	I	I	I	I	0	0	0.01
	3	I	I	I	I	I	I	I	I	I	I	I	0	0	0.01
Hyoscine HBr	1	I	I	I	I	I	I	I	I	I	I	I	0	0	0.01
Papaverine HCl*)	1	0	0												> 40.00
Adiphenine*)	1	I	0	0	0										10.00
Benactyzine	1	I	I	I	I	0	0								1.25
Promethazine	1	I	I	0	0	0	0								5.00
Diphemanil	1	I	I	I	I	0	0								1.25
Oxyphenonium	1	I	I	I	I	I	I	I	I	I	I	0	0		0.02
Butylscopolamine	1	I	I	I	0	0	0								2.50

*) Also tested at doses of 40 and 20 mg/kg, papaverine was found inactive at these doses, whereas adiphenine produced significant inhibition of salivation.

Weaker salivation inhibitory effects with minimal effective doses from 1–5 mg/kg could be demonstrated in benactyzine, promethazine, diphemanil and butylscopolamine. Adiphenine (trasentin ®) had a weak salivation inhibitory effect with a minimal effective dose of 10 mg/kg, corresponding to about 1/500 the activity of atropine. Papaverine was found to be devoid of salivation inhibitory effect at doses up to 40 mg/kg.

Results of bio-assays with anticholinergic reference drugs are summarized in table 3. It is seen that atropine sulphate had a strong salivation inhibitory effect in five assays, the ED50 ranging from 5.5–10 μg/kg. Hyoscine methonitrate was found to be the most potent salivation inhibitory agent with an average ED50 of 3 μg/kg.

The duration of the salivation inhibitory effect produced by a large dose of 50 μg/kg of atropine was also studied. As seen from table 4 the

Table 3

Bioassay of atropine-like compounds on mice for antagonists to pilocarpine-induced salivation.

Compound	Assay number	No. of mice per dose level	Dose levels in μg/kg	Inhibition of salivation, ED50 μg/kg	Mean ED50 μg/kg
Atropine sulphate	1	4	3, 6, 12, 25	7.6	
–	2	4–6	3, 6, 1., 25	9.5	8.0
	3	6	3, 6, 12, 25	10.0	
	4	6	3, 6, 12, 25	7.5	
	5	6	6, 12, 25	5.5	
Hyoscine bromide	1	6	3, 6, 12	6.6	6.0
–	2	6	3, 6, 12	5.6	
–	3	6	3, 6, 12	5.8	
Hyoscine methonitrate	1	6	1.5, 3, 6, 1	2.9	3.0
	2	6	1.5, 3, 6, 12	3.5	
–	3	6	3, 6, 12	2.5	

Test compounds were given c. to anaesthetized mice. Pilocarpine was given s.c. 15 minutes later t dose of 2.0 mg/kg, and the sal vary flow was determined from 0–20 minutes after the pilocarpine injection. Percentage salivary inhibition is calculated by the formula $100 - \frac{TR \times 100}{C}$ where TR is the average salivary spot area per mouse in the group treated with test compound nd C is the verage salivary spot area per mouse of a simultaneously tested control group injected with 0.9% saline. Dose response lines and ED50 values were btained graphically from a plot f log dose against percentage salivary inhibition.

Table 4

Duration of the salivation inhibit ry effect of tropine sulphate in mice.

Atropine sulphate, s.c. dose in mg/kg	Time in min. between atropine and pilocarpine injections	Number of animals	Percentage inhibition of salivary flow
0.05	15	12	97
0.05	30	6	84
0.05	60	6	40
0.05	120	6	23

Collection of saliva was performed 0–20 min. after i jecting 2.0 mg/kg of pilocarpine.

effect was near 100 / when atropine was given 15 minutes before pilocarpine and decreased steadily when the interval between atropine and pilocarpine injections was increased, reaching a value of 23 when the interval was 120 minutes.

Discussion

Methodological

The sensitivity of the method was found to be greatest with a total collection period of twenty minutes, corresponding to the high and constant rate of salivary flow demonstrable during 0–20 minutes after injection of the standard dose of 2.0 mg/kg of pilocarpine. The longer collection period employed in earlier experiments decreased the sensitivity of the procedure.

The fixed time interval of fifteen minutes between injection of the test compound and pilocarpine was also used by BROWN & QUINTON (1957) for rabbits. As the collection period in our mouse method extends from 15–35 minutes after injection of test drug, conditions are optimal only for those drugs possessing peak anticholinergic effect during this period, such as, for example, atropine, hyoscine and hyoscine methonitrate (see also PULEWKA 1932).

Male mice were preferred because their salivation appeared to be more profuse than that of females. The difference was, however significant only at a level of p = 0.2. The mortality of male mice during the ex perimental procedure was about 1 /. In the body weight group of 20–26 g, general anaesthesia with the standard dose of urethane was obtained in 100 / of the mice. Whereas larger laboratory animals usually do not

recover after urethane anaesthesia, mice regained consciousness after 5-7 hours, probably because of their mechanism for rapid elimination. A certain percentage of mice with a body weight of less than 20 g did not reach the stage of general anaesthesia on the standard dose of urethane, and among animals with body weights over 26 g an increased mortality was observed during the test.

Drugs

When the sensitivity of the method is judged with the 50 / salivation inhibitory dose (ED50) of atropine as criterion, it is of the same order of magnitude as that given by the rabbit method (BROWN & QUINTON 1957). These authors obtained an ED50 for atropine sulphate of 3.4–7.5 µg/kg in a total of five assays under comparable conditions on urethane-sedated rabbits. The corresponding values from our method are 5.5–10.0 µg/kg, also for five assays

It may be of interest to compare the method with procedures for other species as to the ratio of activity of known reference drugs. The anti-salivation activity of hyoscine methonitrate in man was found by NYMAN (1943) to be 3.6 times that of atropine. With our method on mice, the activity of hyoscine methonitrate was found to be about three times that of atropine Nyman also found that papaverine in high clinical doses was without effect in man and that adiphenine had an activity corresponding to 1/700 that of atropine These findings correspond also with our own results on mice. BROWN & QUINTON (1957) found that in rabbits the activity of oxyphenonium is roughly similar to that of atropine. Our own experiments with oxyphenonium gave comparable results.

The anti-salivation method on mice also allows us to compare mydriatic and anti-salivation activity of new compounds and to establish an activity ratio for these two anticholinergic effects. This is of practical interest, because the anti-salivation effect may be considered to be closely related to the desirable inhibitory effect of new drugs on gastrointestinal hyper secretion Further atropine sulphate inhibits salivation at considerably lower doses than those required to produce mydriasis. This relation is also seen in man after therapeutic doses of atropine. The doses of atropine necessary to increase the pupil diameter of the mouse to about 50 / of its maximal size ranges from 40 to 160 µg/kg after parenteral administration (QUINTON 1963 PULEWKA 1932 ING *et al* 1945) As shown in the work reported here, the doses of atropine required to produce 50 / inhibition of pilocarpine-induced salivation are 5–20 times lower

It may be mentioned that salivation in anaesthetized mice can be induced also by sympathomimetic drugs. The amount of saliva produced

is, however much smaller than that produced by pilocarpine. Results of some inhibition experiments were similar to those reported by EMMELIN, HOLMBERG & OHLIN (1965) for the rat.

In conclusion it should be emphasized that the advantages of the mouse anti-salivation method are simplicity sensitivity and capacity One complete assay of atropine, comprising tests at four dose levels and including two control groups, on a total of 36 mice, may be performed by one technician on one day It appears that mice, compared with rabbits, do not show greatly varying atropine sensitivity and it is therefore unnecessary to preselect the animals.

Summary

A simple, rapid and sensitive method for estimation of anticholinergic drug effects in anaesthetized mice is described. It is based on the antagonism of atropine like drugs against pilocarpine induced salivation.

The 50 / salivation inhibition doses of atropine sulphate, hyoscine bromide and hyoscine methonitrate were determined and found to be 8.0, 6 0, and 3 0 µg/kg, respectively

Examples of applying the anti-salivation method in mice to the screening of new compounds for anticholinergic activity are given.

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From the Department of Pharmacology University of Göteborg, Göteborg, Sweden

Structure-Activity Relationships for Release of ^{14}C-Octopamine from Adrenergic Nerves by Phenylethylamines

By

Arvid Carlsson and Bertil Waldeck

(Received December 6, 1965)

Tyramine (TA), when injected into animals is rapidly converted to octopamine (OA), and this conversion has been shown to occur in the adrenergic nerves (CARLSSON & WALDECK 1963b FISCHER *et al* 1964) The accumulation of octopamine in the nerves is blocked by reserpine, indicating that this accumulation occurs in the amine storing granules (CARLSSON & WALDECK 1963b) Further evidence in support of this view has been obtained by differential centrifugation (MUSACCHIO, KOPIN & SNYDER 1964). It has also been shown (CARLSSON & WALDECK 1965 see CARLSSON 1965) that the OA is rapidly released by reserpine, prenylamine and (−)-metaraminol In the investigation reported here structure-activity relationships of agents causing such release were studied.

Material and Methods

Mice divided at random into groups of six were used. Equimolar amounts (1 µM/kg) of some phenylethylamine derivatives were given i. 15 minutes after the i. administration of 0 2 mg/kg ^{14}C TA. After another 15 minutes the animals were killed, and the ^{14}C-OA levels in the hearts were determined as previously described (CARLSSON & WALDECK 1963a ALMGREN, ANDÉN & WALDECK 1965)

The drugs used were

(−)-noradrenaline (Sigma Chemical Company) (+)-adrenaline (Dr A. M. Lands, Sterling-Winthrop Res. Inst.) (±)-amphetamine, dopamine (Sigma Chemical C mpany) (−)-ephedrine (E. Merck) epinine (Bios Laboratories) (±)-N-ethylnormetanephrine (effortil ®, Boehringer Ingelheim through ERCO Stockholm) hordenine (E. Merck); 6-hydroxy-

One asterisk indicates individuals and organizations who generously donated drugs.

dopamine (Dr C. A. Stone, Merck, Sharp and Dohme) p-hydroxymethamphetamine (Knoll A. G.) 6-hydroxynoradrenaline isonormetanephrine (±)-isoprenaline (LEO Ltd.) (±)-metanephrine (Winthrop Products) (−)-metaraminol (Dr Henry Merck, Sharp and Dohme) (+)-metaraminol β-methoxydopamine 3-methoxy-ephedrine methoxyphenadrine (ortodrinex ®) 3-methoxytyramine (Dr A. Pletscher, Hoffman-La Roche) α-methyldopamine (Dr A. Pletscher Hoffman-La Roche) *α-methylmetoxytyramine (−)-α-methylnoradrenaline (Farbwerke Hoechst) *α-methyl-normetanephrine α-methyl-m-tyramine (−)-noradrenaline (Fluka A.G.) *(+)-noradrenaline; norephedrine (Merck & Co.) (±)-normetanephrine (Winthrop Products) ( )-m-octopamine (novadral ® Diw g Chemische Fabr) *(±)-p-octopamine orciprenaline (Alupent ® Boehringer Ingelheim through ERCO, Stockholm) m-oxedrine (Winthrop Lab) p-oxedrine phenylethylamine (BDH) m-tyramine (Dr A. Pletscher, Hoffman-La Roche) o-tyramine (Dr A. Pletscher Hoffman-La Roche) and p-tyramine (Biochemica, Roche) Tyramine-^{14}C (p-hydroxyphenylethylamine-1-^{14}C, specific activity 1 83 mC/mM) was obtained from New England Nuclear Corporation, Boston, Mass., U.S.A.

Results

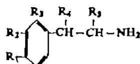
The results are shown in table 1 Although the number of experiments for each compound is small and the scatter relatively large, certain conclusions about structure-activity relationships can be drawn. The observations made were

1) Introduction of a hydroxyl group in the 2 (or 6) position of the ring seemed to cause a slight decrease in activity (compare o-tyramine with phenylethylamine, 6-hydroxydopamine with dopamine and 6-hydroxy-noradrenaline with noradrenaline)
2) Introduction of a hydroxyl group in the 3 position of the ring caused a moderate to strong increase in activity (compare m-tyramine with phenylethylamine, α-methyl-m-tyramine with amphetamine and metaraminol with norephedrine)
3) Introduction of a hydroxyl group in the 4 position of the ring seemed to cause a slight increase in activity (compare p-tyramine with phenylethylamine, p-hydroxymethamphetamine with methamphetamine)
4) Generally the effects of hydroxylation as described under 2) and 3) appeared to be abolished or even reversed when the hydroxylation resulted in formation of a 3,4-dihydroxy derivative (compare dopamine with m-tyramine and p-tyramine, noradrenaline with m-octopamine and p-octopamine, α methyldopamine with α-methyl-m-tyramine, α-methylnoradrenaline with metaraminol compare, however also adrenaline with m-oxedrine and p-oxedrine)

Two asterisks indicate drugs generously supplied by Hässle Ltd., Göteborg, Sweden, through Dr H Corrodi. We gratefully acknowledge these gifts.

Table 1

Release of octopamine in the mouse heart by some phenylethylamine derivatives. [14]C-tyramine 0.2 mg/kg was given i. 15 min. before i. injection of 1 μM/kg of the compound to be tested. After another 15 min. the animals were killed and the hearts removed for analysis. The results are expressed as [14]C-octopamine content as percentages of the mean of 23 control groups, 28 ± 5 g/g (standard deviation). The values are the means of experiments ± standard erro of the means.



Compound tested	R	R2	R3	R4	R5	N-subst.	Per centage f control ± s.e.m.	n
phenylethylamine	–	–	–	–	–	–	77 ± 2	2
p-tyramine	OH	–			–		62 ± 3	3
m-tyramine	–	OH	–				58 ± 10	3
o-tyramine	–	–	OH	–			89 ± 0	2
dopamine	OH	OH	–	–		–	57 ± 5	3
6-hydroxydopamine	OH	OH	6-OH	–	–	–	61 ± 9	2
(±)-p-octopamine	OH		–	OH	–	–	56 ± 5	3
(±)-m-octopamine		OH		OH		–	22 ±	3
(−)-noradrenaline	OH	OH		OH	–	–	46 ± 6	4
(+)-noradrenaline	OH	OH	–	OH		–	53 ± 6	4
(±)-6-hydroxy-noradrenaline	OH	OH	6-OH	OH		–	79	1
α-methyl-m-tyramine	–	OH		–	CH_3	–	37 ± 5	2
α-methyldopamine	OH	OH		–	CH_3		50 ± 0	2
(−)-metaraminol		OH	–	OH	CH_3		3 ± 2	4
(+)-metaraminol		OH		OH	CH_3	–	67 ± 14	3
(−)-α-methylnoradrenaline	OH	OH		OH	CH_3		36 ± 7	3
hordenine	OH	–	–			$(CH_3)_2$	93 ± 11	2
epinine	OH	OH			–	CH_3	54 ± 8	3
p-oxedrine	OH	–	–	OH		CH_3	93 ± 21	2
(−)-m-oxedrine		OH		OH		CH	59 ± 2	2
(±)-N-ethylnormeta-oxedrine		OH	–	OH		CH_3 CH_3	68 ± 21	2
(−)-adrenaline	OH	OH		OH	–	CH	35 ± 9	3
(+)-adrenaline	OH	OH	–	OH		CH_3	64 ± 1	3
(±)-isoprenaline	OH	OH		OH		CH $(CH_3)_2$	103 ± 23	2
orciprenaline		3,5-OH		OH		CH $(CH_3)_2$	84 ± 2	2
p-hydroxymethamphetamine	OH			–	CH	CH_3	61 ± 11	
(±)-amphetamine					CH		107 ± 7	2
methamphetamine			–	–	CH	CH_3	93 ± 4	2
norephedrine		–		OH	CH	–	100	1
(−)-ephedrine				OH	CH	CH_3	89 ± 7	2
3-methoxytyramine	OH	OCH_3	–	–		–	96	1
(±)-normetanephrine	OH	OCH_3		OH	–		114	1
(±)-iso-normetanephrine	OCH_3	OH	–	OH			87 ± 2	2

Table 1 (Continued)

Compound tested	R_1	R_2	R_3	R_4	R_5	N-subst.	Percentage of control ± s.e.m.	n
(±)-metanephrine	OH	OCH_3	–	OH	–	CH_3	104	1
(±)-3-methoxy-norephedrine	–	OCH_3	–	OH	CH_3	–	111	1
β-methoxydopamine	OH	OH	–	OCH_3	–		70 ± 12	2
methoxyphenadrine	–		OCH_3	–	CH_3	CH_3	96	1
α-methylmethoxytyramine	OH	OCH_3		–	CH_3		114	1
(±)-α methyl-normetanephrine	OH	OCH_3	–	OH	CH_3	–	104	1

5) Methylation of the 3-hydroxyl group appeared to abolish the activity (compare 3-methoxytyramine with dopamine, normetanephrine with noradrenaline, metanephrine with adrenaline 3-methoxynorephedrine with metaraminol, α methylmethoxytyramine with α-methyldopamine, and α methylnormetanephrine with α-methylnoradrenaline).
6) Introduction of an hydroxyl group in the β-position on the side chain increased the activity of 3-hydroxy compounds considerably but this appeared to be true only for the laevorotatory enantiomorphs (compare (±)-m-octopamine with m tyramine, (−)-metaraminol and (+)-metaraminol with α-methyl-m-tyramine). In 4-hydroxy and 3,4-dihydroxy compounds the introduction of a β-hydroxy group caused a smaller increase in activity (compare p-octopamine with p-tyramine, noradrenaline with dopamine, α methyl noradrenaline with α-methyl dopamine, adrenaline with epinine) and the difference between the enantiomorphs was slight, if any
7) The introduction of an α methyl group possibly caused a slight increase in activity (compare m-tyramine, m-octopamine, dopamine and noradrenaline with their α methyl derivatives).
8) The effect of N methylation was not uniform However the introduction of an isopropyl group on the nitrogen atom greatly reduced the activity (compare isoprenaline with noradrenaline)
9) Compounds with an unsubstituted phenyl ring were almost devoid of activity at the doses employed

The most striking positive effects were caused by 3-hydroxyl and β-hydroxyl groups and are illustrated by the high activity of (−)-metara-

minol and (±)-m-octopamine. For metaraminol a clearcut stereospecificity in relation to the β-hydroxyl group was also demonstrated.

The most striking negative effects were those caused by blocking the 3-hydroxyl group (by methylation) and by introduction of an isopropyl group on the nitrogen atom

Discussion

The OA-releasing activity of a compound may be assumed to depend on its affinity for the storage mechanism of the granules and on its concentration in the cytoplasm surrounding the granules. This concentration in turn may depend on several factors, namely a) the efficiency by which the compound is transported into the nerve through the cell membrane b) the ability of the compound to diffuse through the cell membrane c) the metabolic stability of the compound and d) other factors affecting the distribution and fate of the compound in the body

The importance of the transport through the cell membrane is demonstrated by the fact that blockade of this transport abolishes the OA and noradrenaline releasing effect of an amine such as (−)-metaraminol (Carlsson & Waldeck 1965a). On the other hand, amines with a high lipid solubility retain their activity after blockade of this transport mechanism, e.g. reserpine and prenylamine. In our investigation the compounds with an unsubstituted phenyl ring were found to be almost inactive This may be due to low affinity for the amine storage mechanism or to the fact that these compounds possess a high lipid solubility and thus cannot be concentrated by the amine transport mechanism of the cell membrane ("the amine pump") even if they are transported efficiently through the cell membrane, diffusion in the outward direction will prevent the building up of a high concentration in the cytoplasm. The introduction of hydroxyl groups into the ring reduces the lipid solubility and thus indirectly increases the concentrating power of the "amine pump" This may explain the positive influence of ring hydroxylation on amine releasing activity but does not account for the specificity of hydroxyl group position. Whether the especially high activity of 3-hydroxy compounds is due to efficient transport through the cell membrane or to high affinity for the granules cannot be decided from present information. The available evidence suggests, however that the structural requirements of the amine storage mechanism of the granules are more stringent than those of the "amine pump of the cell membrane. That a certain degree of structural specificity exists, however is indicated by the fact that the

amine pump" can hardly be utilized by isoprenaline and 5-hydroxytryptamine and that it is more efficiently blocked by imipramine derivatives with one rather than two methyl groups on the side chain nitrogen atom (MALMFORS 1965 CARLSSON & WALDECK 1965b CORRODI, MALMFORS & SACHS 1965 1966)

There seems to be little doubt that the stereospecificity observed in this study resides in the amine storage mechanism of the granules. MALMFORS (1965) observed no difference between the transport through the cell membrane of the two enantiomorphs of noradrenaline. That the affinity for the granules is greatly influenced not only by the β-hydroxyl group, as shown by the stereospecificity but also by the phenolid hydroxyl groups, is indicated by the fact that octopamine is much more easily released from the granules than noradrenaline (CARLSSON & WALDECK 1965a MUSACCHIO, KOPIN & WEISE 1965) The observations of MUSACCHIO *et al* in their studies on intracellular distribution of different amines and on their release *in vivo* suggest that the affinity for the granules decreases with decreasing number of hydroxyl groups in the order noradrenaline > dopamine > octopamine > tyramine In our investigation the averages obtained for these four amines were 46, 57 56 and 62, respectively Significant differences were thus hardly observed. This suggests that *in vivo* factors operate that tend to neutralize the effect of different affinities for the granules. One such factor might be metabolic stability For example, circulating noradrenaline is rapidly metabolized, mainly through the action of liver catechol-O-methyl transferase (AXELROD *et al* 1958 AXELROD & TOMCHICK 1958 CARLSSON & WALDECK 1963a), so that less noradrenaline will become available for uptake by the nerve endings. However the possibility must also be considered that increasing the number of hydroxyl groups will reduce the affinity for the carrier system of the amine pump of the cell membrane There are certain indications in favour of this assumption In experiments on isolated rat hearts, the uptake and accumulation of noradrenaline, when added at a low concentration to the perfusion fluid, was blocked less efficiently by phenylethylamines possessing a β-hydroxyl group than by those devoid of it (BURGEN & IVERSEN 1965) It does not seem very probable that under these conditions metabolic conversion of the compounds can be an important factor

Summary

Forty phenylethylamine derivatives were tested for their ability to release ^{14}C-octopamine (formed from ^{14}C tyramine) from mouse heart. The potency was greatly increased by the introduction of a phenolic 3-hydroxyl group and an aliphatic β-hydroxyl group. Stereospecificity was demonstrated for the β-hydroxyl group. Blockade of the 3-hydroxyl group (by methylation) or introduction of an isopropyl group on the nitrogen atom abolished the activity. The findings are discussed in relation to the amine uptake and storage mechanisms of the adrenergic nerve.

Acknowledgements

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From the Departments of Pharmacology and Psychology, University of Göteborg and the Department of Histology, Karolinska Institutet, Stockholm, Sweden

Functional Role of the Nigro-Neostriatal Dopamine Neurons*)

By

Nils-Erik Andén, Annica Dahlström, Kjell Fuxe and Knut Larsson

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In previous investigations it has been found that the dopamine (DA) of the neostriatum (the caudate nucleus and putamen) is present in nerve terminals belonging to special neurons with the cell bodies in the substantia nigra – mainly in its zona compacta – and with the non-terminal axons ascending in the crus cerebri and the internal capsule (ANDÉN *et al.* 1964, BERTLER *et al.* 1964, ANDÉN *et al.* 1965 a & b). These nigro-neostriatal DA neurons do not cross the midline, and it should therefore be possible to investigate their functional role after a unilateral lesion. In the work reported here, such a study was performed in rats both with and without drugs that interfere with DA metabolism.

Materials and Methods

About 200 adult Sprague-Dawley or hooded rats were used. Two types of operations were performed. In the first, the DA pathway was unilaterally destroyed by electrolytic lesion (HILLARP 1947) in the crus cerebri at the level of the corpus mamillare, and the animals were examined 10 to 60 days after the operation. In the second, the corpus striatum was removed unilaterally by suction with a fine glass cannula under ether anaesthesia, and the animals were examined 8 to 24 hours afterwards. The effect of the lesion on the nigro-neostriatal DA neurons was determined in the first series by one of two biochemical analyses: (1) determination on each side of the brain of the DA in the corpus striatum and the DA and noradrenaline in the rest of the telencephalon plus the diencephalon (CARLSSON & WALDECK 1958, BERTLER, CARLSSON & ROSENGREN 1958); (2) determination on each side of the brain of the 3,4-dihydroxyphenylalanine (L-DOPA) decarboxylase activity *in vitro* in the corpus striatum and in the other parts of the telencephalon plus the diencephalon (BERTLER & ROSENGREN 1959). The second type of lesion was investigated by

*) Results presented in part at the International Symposium on "Mechanisms of Release of Biogenic Amines" in Stockholm, February 23, 1965.

Table 1

Drugs and doses	Number of rats examined with a unilateral lesion of the nigro-neostriatal dopamine pathway in the	
	crus cerebri	corpus striatum
Reserpine 10 mg/kg i.p.	20	10
Haloperidol 2 mg/kg i.p.	20	10
Chlorpromazine 10 mg/kg i.p.	3	6
2.5 mg/kg i.p.	2	6
Promethazine 20 mg/kg i.p.	3	3
Phenoxybenzamine 20 mg/kg i.p.	3	5
10 mg/kg i.p.	3	5
Propranolol 0.5–12 mg/kg i.p.	3	3
Pentobarbital sodium 8–40 mg/kg i.p.	1	3
Nialamide (100 mg/kg i.p.) 2 hours before reserpine (10 mg/kg i.)	10	10
Nialamide (100 mg/kg i.p.) 2 hours before L DOPA 25 mg/kg i.v.	5	
50 mg/kg i.		10
75 mg/kg i.	5	10
Nialamide (100 mg/kg i.p.) 2 hours before DL 5-hydroxytryptophan 25 mg/kg i.	4	8
50 mg/kg i.	1	8

macroscopical observation of the brains after death In all the animals considered in this paper more than two thirds of the corpus striatum had been removed.

The animals were treated with drugs that interfere with the monoamine metabolism (see table 1). Since many of the drugs used cause a disturbance in body temperature regulation, care was taken to prevent hypothermia in the rats.

Most of the lesions were made on the left side, but for control some were made on the right, when all results were reversed, as expected.

Results

Biochemistry

A lesion in the crus cerebri caused in most animals a loss of 60% or more of the DA or DOPA decarboxylase activity in the ipsilateral corpus striatum compared with the opposite side. In five rats there was no detectable disappearance of DA or DOPA decarboxylase activity and these rats never displayed any asymmetry (see below). About half of the rats had lost a little of the noradrenaline or DOPA decarboxylase activity in some or all forebrain areas outside the corpus striatum, but there was no correlation between this finding and the asymmetries described below

Table 2

Effect of drugs intended to block or facilitate monoamine transmission on the turning of rats in relation to a unilateral lesion of the nigro-neostriatal dopamine pathway

Drug	Turning in relation to the lesion
No drug treatment	No turning
Blockade of monoamine transmission	
Reserpine	Contralateral
Haloperidol	Contralateral
Chlorpromazine	Contralateral
Promethazine	No turning
Phenoxybenzamine	No turning
Propranolol	No turning
Pentobarbital sodium	No turning
Facilitation of monoamine transmission	
Nialamide + reserpine	Ipsilateral
Nialamide + DOPA	Ipsilateral
Nialamide + 5-hydroxytryptophan	No turning

Position and movements (table 2)

Without drug treatment

After the crus cerebri lesions all the animals after a successful operation showed a slight deviation of the tail root to the same side as the lesion (fig. 1). Sometimes it was also possible to observe a turning of the head and of the whole tail to the operated side, and these phenomena were mainly seen after large lesions and during the first 2–3 postoperative days. After removal of the corpus striatum, no significant asymmetries could be seen except that most of the animals turned the tail and head towards the operated side.

Drugs intended to block monoamine transmission

Reserpine After both types of lesion, reserpine administration caused the rats to turn tail and head to the side opposite to the lesion, to abduct the extremities, especially the hindlegs, on the operated side and to adduct the extremities below the trunk on the unoperated side (fig. 2). The syndrome began to appear about one hour after the intraperitoneal injection of 10 mg/kg body weight and was completely developed after two hours. Usually it was possible to feel a rigidity of the extremities on the



Fig. 1 Rat with a lesion of the left nigro-neostriatal dopamine pathway produced in the crus cerebri 30 days before.

unoperated side On this side there was also a more pronounced tremor than on the operated side, with a frequency of about 20 per second. The asymmetries could still be seen after 24 hours, but were then much weaker and reinjection of the same dose of reserpine did not increase them.

One hour after an intraperitoneal injection of 10 mg/kg reserpine, when the effects appeared, there was still about 25 / of the normal amount of DA in the corpus striatum on the unoperated side. After two hours, *practically no DA could be found.*



Fig. 2. Reserpine treatment (10 mg/kg i.p. 3 hours before) of a rat with a lesion of the left nigro-neostriatal dopamine pathway produced in the crus cerebri 30 days before.

In two rats with electrolytic lesions in the crus cerebri there was no significant reduction in DOPA decarboxylase activity of the corpus striatum on the operated side. These animals showed no clear asymmetries after reserpine treatment. As a control for the effects obtained after removal of the corpus striatum, one rat was examined after suction removal of only the neocortex overlying the corpus striatum. This rat showed no asymmetries after reserpine treatment.

Haloperidol The same turning of the tail, head and extremities as after reserpine treatment was observed after the administration of haloperidol (2 mg/kg i.p.) The syndrome appeared earlier and was shorter lasting than after a reserpine injection. No tremor could be seen, not even after 20 mg/kg i.p. Haloperidol did not produce any asymmetry after the removal of only the neocortex overlying the corpus striatum (1 rat). Neither were any asymmetries produced in those rats in which the crus cerebri lesions did not cause any reduction in DA content on the operated side (3 rats).

Chlorpromazine Treatment with chlorpromazine (2.5 to 10 mg/kg i.p.) caused asymmetries similar to those produced by haloperidol. The signs followed about the same time course after the two drugs. After 10 mg/kg i.p. the hindlegs were often slightly paretic, but even after this dose no tremor could be detected.

Promethazine This phenothiazine derivative, though structurally related to chlorpromazine, did not give any asymmetry despite the production of a pronounced sedation. Chlorpromazine was always given subsequently to the same rats and was found to produce the effects described above.

Phenoxybenzamine and propranolol Since chlorpromazine blocks the peripheral adrenergic receptors of the α-type, a potent blocking agent of these receptors, i.e. phenoxybenzamine (10–20 mg/kg i.p.), was also tested. In spite of deep sedation produced by this, little if any asymmetry could be seen in animals with a crus cerebri lesion. Nor did propranolol (inderal ®), a blocking agent of the adrenergic β-receptors, produce any asymmetry in doses increased stepwise from 0.5 to 12 mg/kg i.p. In all these animals haloperidol or chlorpromazine given afterwards produced the usual effects.

Pentobarbital Pentobarbital sodium (nembutal ®) was given in doses that were increased stepwise from 8 mg/kg i.p., which caused only slight sedation, to 40 mg/kg i.p., which caused deep anaesthesia. No asymmetry was ever observed. In fact pentobarbital sodium (40 mg/kg i.p.) abolished the asymmetries obtained after treatment with reserpine (10 mg/kg i.p., 1 rat) or after treatment with nialamide plus reserpine (100 mg/kg i.p. 2 hours before 10 mg/kg i.v. 1 rat).

Drugs intended to facilitate monoamine transmission

Nialamide plus reserpine A monoamine oxidase inhibitor at a low dose does not by itself produce any pharmacological effects during the first few hours after the administration, e g. nialamide 100 mg/kg i.p 2 hours. Such treatment, however produces an inhibition of the enzyme sufficient to reverse the reserpine syndrome. The reason for this effect is that the amines released from the storage granules are not degraded inside the nerve terminals, since the monoamine oxidase is inhibited. Therefore the amines can leak out through the axon membrane and reach the receptors in free and active form. Within five minutes after an intravenous injection of reserpine (10 mg/kg i.v) given to rats pretreated with nialamide (100 mg/kg i p., 2 hours) the animals began to rotate vigorously to the operated side. They continued to do so for several hours, until they were completely exhausted During the few moments when the movements stopped it was clearly seen that the tail and head were turned to the operated side the extremities on the side opposite to the lesion were abducted, whereas those on the lesion side were adducted below the trunk, i.e the body position was completely the reverse of that observed after reserpine treatment alone (fig. 3) Of the animals with the corpus striatum removed, mainly those turning spontaneously to the unoperated side were chosen for this type of experiment.

There was no asymmetry after treatment with nialamide plus reserpine in one rat, in which only the neocortex overlying the corpus striatum had been removed by suction.

Haloperidol (2 mg/kg i p) was given to a few rats treated with nial-



Fig. 3 Reserpine treatment (10 mg/kg i. 30 minutes before) after monoamine oxidase inhibition (nialamide 100 mg/kg i.p. 2 hours before the reserpine injection) of a rat with lesion of the left nigro-neostriatal dopamine pathway produced in the crus cerebri 10 days before.

amide plus reserpine, and these ceased to turn to the operated side some were found even to turn to the opposite side.

L DOPA When L DOPA (50–75 mg/kg i v) was given to rats pretreated with the monoamine oxidase inhibitor nialamide (100 mg/kg i p 2 hours before) the animals began to rotate to the operated side within 10–15 minutes. The rotation was usually not as continuous as with nialamide plus reserpine and sometimes the animals only jumped in circles to the operated side Most of the animals had been injected with reserpine (10 mg/kg i.p) 2–4 hours before nialamide (100 mg/kg i p), and so they were forced to turn to the unoperated side at the time of the L DOPA injection. Treatment of these rats with L DOPA, thus, produced a change in the direction of turning from the contralateral to the ipsilateral side of the lesion

The DOPA effect was usually more pronounced in the rats after removal of the corpus striatum than after a crus cerebri lesion. With the latter case animals often turned to the unoperated side after small doses (25 mg/kg i.v) or at first after larger ones (50–75 mg/kg i.v) As there was always a formation of DA in the denervated corpus striatum, although much smaller than in the intact one, this phenomenon may be due to a denervation supersensitivity

5-Hydroxytryptophan When DL 5-hydroxytryptophan (25–50 mg/kg i.v) was given after nialamide (100 mg/kg i.p 2 hours before), no asymmetry could be seen, despite the presence of such effects as tremor and hyperextension Nor could DL 5-hydroxytryptophan change the asymmetry produced by pretreatment with reserpine (10 mg/kg i p 2–4 hours before nialamide) Two of the rats were treated with L DOPA (75 mg/kg i.v) when the tremor and hyperextension had subsided, and these animals then began to rotate to the operated side

Discussion

The aim of the investigation was to study the function of the nigro-neostriatal DA neurons. In all likelihood, however the lesions produced did not interfere exclusively with the pathways to and from the corpus striatum. For example, it is conceivable that both types of lesions interrupted nerve tracts from or to the neocortex. It is therefore possible that the asymmetries were due to an action of the drugs on neocortical noradrenaline transmission mechanisms. This noradrenaline is present in nerve terminals belonging to neurons whose cell bodies are in the pons and the medulla oblongata (ANDÉN *et al* 1966) and whose axons ascend in the medial forebrain bundle (ANDÉN *et al* 1965b) For this reason a

few experiments have been performed after unilateral lesions at the junction between the pons and the mesencephalon or in the medial forebrain bundle at the level of the anterior hypothalamus, after which the neocortical noradrenaline but not the neostriatal DA had disappeared (ANDÉN DAHLSTRÖM, FUXE, LARSSON OLSON & UNGERSTEDT unpublished results) The administration of reserpine, haloperidol or nialamide plus reserpine to rats with such lesions did not produce any detectable asymmetry It is therefore probable that the asymmetries were due to interference with the function of the dopamine in the corpus striatum. That cortical mechanisms are also involved cannot, however be excluded.

According to our unpublished results the rat corpus striatum contains much more DA than 5-hydroxytryptamine, about 6 µg/g and 0.3 µg/g tissue respectively The amount of noradrenaline is negligible (cf SHARMAN & VOGT 1965) Most of the drugs used in this investigation may affect the function of both DA and *5-hydroxytryptamine* It should therefore be of interest that there is an asymmetry only after administration of the DA precursor DOPA and not of the 5-hydroxytryptamine precursor 5-hydroxytryptophan This finding suggests that the phenomena observed are due solely to an interference with the neostriatal DA. This view is supported by studies showing that no certain disappearance of the striatal 5-hydroxytryptamine can be detected after our crus cerebri lesions (results not shown)

It is tempting to explain the similar effects of reserpine, chlorpromazine and haloperidol by a common mechanism. As is well known, reserpine produces a blockade of monoamine transmission by depletion of the transmitter store (CARLSSON *et al* 1957 MUSCHOLL & VOGT 1958). Both chlorpromazine and haloperidol reduce the central effects of DOPA and monoamine oxidase inhibitors, in all likelihood in consequence of a blockade of catecholamine receptors. Chlorpromazine is also a fairly potent blocking agent of the peripheral adrenergic α-receptors. Therefore it is reasonable to assume that the turning of the rats to the side opposite to the lesion is due to a presynaptic blockade of neostriatal DA transmission after reserpine and a postsynaptic one after chlorpromazine or haloperidol. The DA receptors are probably not of a pure α-type since there was at most a weak asymmetry after phenoxybenzamine, which blocks the peripheral adrenergic α receptors more effectively than chlorpromazine and in particular than haloperidol The finding that there is no asymmetry after promethazine, pentobarbital or phenoxybenzamine, despite a severe sedation with each of them, shows that the sedation is not in itself of importance. The asymmetries appear to be produced only by drugs with a neuroleptic action, *i e* sedation without anaesthesia. In this connection it is to be noted that reserpine, chlorpromazine and

haloperidol, but not the other drugs, produce an increased concentration of DA metabolites in the corpus striatum (CARLSSON & LINDQVIST 1963 ANDÉN ROOS & WERDINIUS 1964) The explanation for these results may be that the blockade of DA transmission – either presynaptic or post synaptic – results in activation of the nigro-neostriatal DA neurons with consequent increased rate of synthesis of the transmitter

Our experiments show that there is at most only a slight asymmetry after unilateral lesion of the nigro-neostriatal DA pathway The drug-induced blockades and facilitations of DA transmission in such animals are, however accompanied by asymmetries. Since these asymmetries are opposite to each other it seems as though both syndromes are evoked from the intact corpus striatum. This structure is the only place where a DA excess can reasonably be held to exist. Among several possible explanations of these findings the following may be the most plausible. The neostriatum contains a high concentration of acetylcholine (MAC INTOSH 1941), choline acetylase (FELDBERG & VOGT 1948) and acetylcholine esterase (BURGEN & CHIPMAN 1951) It is thus highly likely that the fibres containing acetylcholine esterase in the neostriatum (KOELLE 1954) represent true acetylcholine nerve terminals. The acetylcholine fibres probably ascend uncrossed in the crus cerebri and in the internal capsule, since there is a severe loss of the ipsilateral acetylcholine esterase activity in the neostriatum after unilateral lesions at the junction between the mesencephalon and the diencephalon (SHUTE & LEWIS 1963). In view of these findings, it is probable that the neostriatal acetylcholine terminals were also removed after our crus cerebri lesions, and in fact we have observed a certain reduction of the acetylcholine esterase activity in the ipsilateral neostriatum in a few preliminary experiments of this kind. It is not unlikely that the postulated cholinergic neurons to the neostriatum are functional antagonists of the DA neurons, since atropine and other cholinergic blocking agents are useful in the treatment of parkinsonism. Naturally the lesion may have included an efferent pathway from the corpus striatum or another afferent neuron system to the neostriatum antagonistic to the DA one.

The most constant anatomical lesion in parkinsonism is a disappearance of the cell bodies in the substantia nigra and the most constant biochemical lesion a loss of DA from the neostriatum and the substantia nigra (for references, see ANDÉN *et al* 1964). These changes are probably due to a degeneration of the nigro-neostriatal DA neurons (ANDÉN *et al* 1964). In hemiparkinsonism the cell bodies (GREENFIELD 1958) and the DA (BAROLIN, BERNHEIMER & HORNYKIEWICZ 1964) have disappeared on the side *opposite to the clinical signs* A hemiparkinson-like syndrome can also be seen in monkeys after mesencephalic lesions producing changes

in the magnocellular part of the zona compacta of the substantia nigra (WARD McCULLOCH & MAGOUN 1948 PETERSON *et al* 1948, POIRIER 1960), where the DA cell bodies are localized (ANDÉN *et al* 1964 ANDÉN *et al* 1965a) Recently it has also been demonstrated that such a lesion in monkeys causes a loss of DA from the ipsilateral neostriatum (SOURKES & POIRIER 1965)

The rats with a unilateral lesion of the nigro-neostriatal DA pathway and treated with reserpine display signs similar to those in hemiparkinsonism of primates. If the assumptions mentioned above are correct, the effect is due to a loss of both the DA and the acetylcholine pathway to the neostriatum on the operated side and a blockade by reserpine of the DA transmission on the unoperated side, so that the acetylcholine effect will predominate on this side There seems, however to be one difference between the signs in primates and rats in primates the rigidity and the tremor occur on the contralateral side to the anatomical and biochemical changes, whereas in rats they appear on the same side as the presumed imbalance between neostriatal DA and acetylcholine mechanisms. This apparent species difference may be explained if the impulses to the motoneurons do not descend in the same nerve tracts. The parkinsonian syndrome in primates is dependent on an intact pyramidal tract (see BUCY 1958) Since the pyramidal pathway is almost completely lacking in the rat (DOUGLAS & BARR 1950), the impulses from the corpus striatum may be mediated by other nerve tracts, which are possibly uncrossed.

Summary

The function of the dopamine in the neostriatum (the caudate nucleus and putamen) of rats has been studied after unilateral lesions of the uncrossed nigro-neostriatal dopamine neurons in the crus cerebri or after unilateral removal of the corpus striatum. At most small changes were seen in the operated animals untreated with drugs interfering with the dopamine metabolism. After injection of drugs causing a blockade of dopamine transmission (reserpine, haloperidol, chlorpromazine), the rats turned to the unoperated side after reserpine there was also augmentation of the tremor on the unoperated side Phenoxybenzamine, propranolol, promethazine and barbiturates did not cause any asymmetry With facilitation of dopamine transmission produced by nialamide plus reserpine or L DOPA the rats rotated to the operated side. No asymmetries were observed after 5-hydroxytryptophan It is suggested that the lesions had also affected a nerve tract to the neostriatum, maybe an acetylcholine one, antagonistic to the dopamine pathway

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From the Department of Pharmacology University of Bergen,
Haukelandsveien 10 Bergen, Norway
(Professor dr med. Tollak B. Sirnes)

The Decarboxylation of Some Phenolic Acids by the Rat

By

Ronald R. Scheline

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The presence of pyrogallol in human urine was reported by TOMPSETT (1958), who suggested that it is probably derived from gallic acid (3 4 5-trihydroxybenzoic acid) by decarboxylation in the alimentary tract. However BOOTH *et al.* (1959) detected pyrogallol in the urine of rats after intraperitoneal injection of gallic acid, and they believed this to indicate that decarboxylation is not dependent on passage through the gastrointestinal tract. The decarboxylation of a related compound, protocatechuic acid (3 4-dihydroxybenzoic acid), has been reported to occur in rat faecal and caecal extracts (BOOTH & WILLIAMS 1963b) although this reaction has apparently not been observed after administration of protocatechuic acid to animals.

In view of the paucity of information on this subject, the investigation reported here was undertaken to study the decarboxylation of gallic and protocatechuic acids in rats. The presence of some phenolic compounds in normal urine will also be discussed in relation to our findings.

Methods

Animals. The rats used in these experiments were adult albino males weighing from 250 to 350 g. They were fed on rat diet obtained from Felleskjøpet, Oslo, and were allowed free access to food and water before and during all experiments. Light ether anaesthesia was used when the compounds were given by the oral (stomach tube) or intraperitoneal route, and nembutal sodium (pentobarbital), 35 mg/kg intraperitoneally was used as anaesthetic in the biliary studies.

Chemicals. The phenolic compounds were obtained from commercial sources, except for 3,5-dihydroxy-4-methoxybenzoic acid and 2-O-methylpyrogallol, which were prepared by the methods of SCHÖPF & WINTERHALDER (1940) and GEISSMAN & MOJE (1951) respectively

Metabolic studies. The compounds were given at a dose level of 100 mg/kg unless otherwise stated. The urine and faeces were collected separately in containers placed in solid carbon dioxide by means of the separator and metabolism cage of NIAA (1963). The biliary studies were carried out as previously described (SCHELINE & LONGBERG 1965), except that the bile and urine were collected for 20–24 hours in test tubes maintained at 0°. The 4-hour urine samples after thawing were filtered, diluted to 100 ml and divided into two equal portions, and each was acidified with 5 ml conc. HCl. One portion was immediately extracted with two 50 ml portions of ether and the other was refluxed for 1 hour before

Table I

Rf values and colour reactions of gallic and protocatechuic acids and related compounds.

Compound	Rf value × 100 in solvent system				Colour with		
	A	B	C	D	Fast blue B salt	Diazotized sulphanilamide	Gibbs reagent
Gallic acid	0	0	0	55	pink brown	green-brown	grey-brown
Protocatechuic acid	7	70	8	78	white-tan	pink-tan	grey
Vanillic acid	90	33	95	90	pink-brown	orange	blue
3,5-Dihydroxy-4-methoxybenzoic acid	9	30	12	80	red-purple	brown-yellow	purple
Pyrogallol	5	0	5	69	pink-brown	brown	brown
1-O-Methylpyrogallol	68	66	88	86	white-grey	pink-grey	purple
2-O-Methylpyrogallol	51	72	82	91	violet	yellow-grey	grey-purple
Resorcinol	14	74	15	90	red-purple	yellow-grey	violet-purple
Catechol	39	64	57	90	pink-grey	pink-grey	blue-purple

Solvent system

A benzene-glacial acetic acid-H_2O (6 : 7 : 3, upper phase)

B n-butanol-ethanol-ammonia 0.88 (1 : 1 : 1)

C chloroform-glacial acetic acid-H_2O (3 : 1 : 1, lower phase)

D n-butanol-glacial acetic acid-H_2O (4 : 1 : 5, upper phase)

Spray reagents

Fast Blue B salt

Spray with 0.5 % aqueous solution of the stabilized diazotate of dianisidine and then saturated sodium bicarbonate solution.

Diazotized sulfanilamide

Mix equal volumes of 1 % sulfanilamide in 4 N HCl and 5 % aqueous $NaNO_2$ and extract with 4 volumes of n-butanol. Spray with butanol phase and then saturated sodium bicarbonate solution.

Gibbs reagent

Spray with 0.25 % 2,6-dichloroquinone-4-chloroimide in ethanol and then saturated sodium bicarbonate solution.

ether extraction. Gallic and protocatechuic acids are stable under these conditions of hydrolysis. The ether extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness, and the residue was dissolved in 1 ml acetone. The entire 24-hour bile sample was diluted to 50 ml and acidified with 5 ml conc. HCl. This was extracted three times with 50 ml portions of ether refluxed for 1 hour and then re-extracted twice with ether. The ether extracts were then treated as described above.

In vitro studies. The medium consisted of 0.1 M phosphate buffer (pH 7.4) containing 0.5% glucose, 0.5% yeast extract (Difco) and 0.5% peptone (Difco). Fresh normal rat faeces (20 g) were mixed in a Servall Omni-Mixer with 100 ml medium and centrifuged at low speed. One ml of the supernatant was added to 10 ml medium and 10 mg test substance (1 ml) in a test tube. The tube was flushed with nitrogen, stoppered and incubated at 37°. After incubation (for approximately 22 hours) the sample was acidified with 1 ml conc. HCl and extracted with two 25 ml portions of ether. The ether extract was dried over anhydrous Na_2SO_4, the ether was evaporated and the residue was dissolved in 1 ml acetone. Preliminary experiments were carried out with 1 ml portions of the entire small intestine, caecum or colon contents of a rat after mixing with 10 ml medium. Larger cultures (400–1000 ml) were used to isolate the metabolites of the various phenolic compounds. The compounds were added at a level of 0.1% was described above, but the amount of faeces extract was reduced to 2.5 ml/100 ml medium. After incubation, acidification and continuous ether extraction, the metabolites were isolated by chromatography on large sheets of Whatman no. 3 filter paper.

Chromatographic methods. The acetone extracts of the samples, together with appropriate standards, were examined by thin layer chromatography on 0.5 mm thick plates of cellulose (MN 300, Macherey Nagel and Co.). Rf values of gallic and protocatechuic acids and related compounds and their colour reactions are shown in table 1. Solvent D was used to detect small amounts of gallic acid in pyrogallol.

Results

An initial study of the ability of rat intestinal contents to metabolize gallic and protocatechuic acids showed that these were decarboxylated to pyrogallol and catechol, respectively, when the test substance was added to the medium containing extracts of caecal or colon contents. However, the acids were recovered essentially unchanged when small intestine contents were used. Extracts of normal faeces also readily decarboxylated these phenolic acids, and such extracts were used in subsequent studies. Decarboxylation occurred under anaerobic conditions, and no pyrogallol or catechol was formed when the samples were aerated. No methylated metabolites of gallic or protocatechuic acid were formed when the acids were incubated with extracts of intestinal contents or faeces.

An additional phenolic compound was formed in the gallic acid samples incubated with faeces extracts, which was either absent or present only in trace amounts in the experiments with caecal or colon contents. The compound had Rf values and gave colour reactions identical with those of resorcinol. This finding was confirmed on isolating the metabolite by

preparative paper chromatography and measuring its absorption and fluorescence spectra in 0.01N-HCl The isolated compound and authentic resorcinol showed identical ultraviolet absorption curves with maxima at 273 mμ. Fluorescence measurements with an Aminco-Bowman Spectrophotofluorometer gave fluorescence maxima of 308 mμ for both compounds. Incubation with pyrogallol also resulted in the formation of resorcinol but no methyl derivative Catechol was similarly isolated from a protocatechuic acid sample. This substance and authentic catechol in 0 01N-HCl both gave ultraviolet absorption maxima at 274 mμ and fluorescence maxima at 317 mμ

The inhibition of the decarboxylation reaction by a number of antibiotics was studied by using benzyl penicillin, oxytetracycline, chloramphenicol, streptomycin and neomycin They were added to give a final concentration of 1 or 10 μg antibiotic per ml of sample. The most effective inhibition was seen with oxytetracycline, which completely blocked the decarboxylation at a concentration of 10 μg/ml. Only traces of pyrogallol or catechol were found when 1 μg/ml was used, and the chromatograms of these samples showed prominent gallic or protocatechuic acid spots. Partial inhibition was produced by chloramphenicol, penicillin and neomycin, but streptomycin had no effect.

Chromatograms of urine extracts from four rats given protocatechuic acid orally showed the presence of this compound and vanillic acid in both the non-hydrolysed and hydrolysed samples, with increased amounts in the latter Another prominent spot seen in the hydrolysed samples corresponded in Rf values and colour reactions to catechol. This substance was also usually seen in small amounts in the acid-hydrolysed urines of control rats After administration of protocatechuic acid, the catechol spots on the chromatograms were greatly increased in size, and preliminary experiments at dose levels of 40 and 400 mg/kg showed that this increase paralleled the increasing dose level

The chromatograms of extracts of hydrolysed urine from three rats given protocatechuic acid intraperitoneally showed that the urinary output of catechol was not increased over that in untreated animals. Protocatechuic acid was found in the urine largely in the free state after intraperitoneal dosage. Vanillic acid was excreted partly free and partly conjugated, as it was detected in larger amounts in the extracts of the hydrolysed urines Biliary excretion was studied in one rat given protocatechuic acid intraperitoneally Traces of unchanged compound and vanillic acid were seen on the chromatograms of the unhydrolysed bile extract After hydrolysis, a prominent spot due to vanillic acid and a small spot corresponding to protocatechuic acid were detected.

The chromatograms of non-hydrolysed urine from four rats given gallic acid orally showed two spots not seen in the control samples. These corresponded to gallic acid itself and 3 5-dihydroxy-4-methoxybenzoic acid. Both of these compounds were found in increased amounts in the hydrolysed samples. Also observed on the chromatograms of hydrolysed samples were two spots corresponding to pyrogallol and 2-0-methylpyrogallol. These were not observed on chromatograms of extracts of normal rat urine. Dosing with 30 and 300 mg/kg showed that excretion of the decarboxylated metabolites increased with increasing dose The 24–48 hour urines of the treated rats were free from gallic acid or its metabolites. Intraperitoneal injection of gallic acid in four rats resulted in the urinary excretion of gallic acid and 3,5-dihydroxy-4-methoxybenzoic acid How ever neither pyrogallol or 2-0-methylpyrogallol was detected in any of these urines.

Chromatograms of the extracts of non-hydrolysed 24-hour bile samples from three rats given gallic acid orally showed that gallic acid or its metabilites were not present. Small amounts of 3,5-dihydroxy-4-methoxy benzoic acid were found in the hydrolysed extracts, however and in one experiment a trace of gallic acid was detected. After intraperitoneal injection of gallic acid into two rats, small amounts of 3,5-dihydroxy-4-methoxybenzoic acid were detected in the hydrolysed bile extracts. A trace of gallic acid was detected in one of these.

As a result of the *in vitro* experiments, which indicated that gallic acid and pyrogallol could be metabolized to resorcinol, the chromatograms of urine extracts were carefully checked for its presence. It was often absent or seen only in traces on the chromatograms, but occasionally the hydrolysed urine extracts gave a spot having the Rf values and colour reactions of resorcinol

The metabolism of pyrogallol was studied in two groups of two rats after its oral or intraperitoneal administration. Chromatograms of the extracts of non-hydrolysed 24-hour urines showed no pyrogallol or 2-0-methylpyrogallol. After hydrolysis, however prominent spots corresponding to pyrogallol and 2-0-methylpyrogallol were observed in both groups. Traces of resorcinol were detected in the hydrolysed extracts. No 1-0-methylpyrogallol was found in any of the extracts after dosing with gallic acid or pyrogallol.

The effect of antibiotic treatment on the metabolism of the phenolic acids in rats was compared with the inhibitory effect on decarboxylation found in the *in vitro* experiments Two animals were given the normal diet, and three were given the diet containing 1 / antibiotic two days before and one day after oral administration of the test compound. The

24 hour urines were examined in the normal manner When gallic acid had been given, the chromatograms of the urines from the control rats showed the typical pattern of metabolites whereas those from neomycin-treated animals showed a complete lack of the decarboxylated metabolites, pyrogallol and 2-0-methylpyrogallol Essentially similar results were seen when oxytetracycline was given, although the hydrolysed urine from one treated rat contained a trace of 2-0-methylpyrogallol Protocatechuic acid was administered to another group of normal and neomycin-treated rats. The urines from two animals from the latter group contained no catechol. Catechol was detected in the urine of the third rat, but the amount was less than that seen in the controls No differences in methylation of the acids were observed between the control and antibiotic treated groups.

Discussion

The metabolic fate of protocatechuic acid has been reported by DODGSON & WILLIAMS (1949), who found that it was excreted to a large extent unchanged, but partly combined with glucuronic and sulphuric acids, by rabbits DEEDS *et al* (1955 & 1957) showed that it was also methylated in rats and rabbits to vanillic acid (4-hydroxy 3-methoxybenzoic acid). DACRE & WILLIAMS (1962) studied the fate of ^{14}C labelled protocatechuic acid in rats and found that 70 / of the radioactivity was excreted in the urine in seven days after oral dosage (100 mg/kg) Most of the radioactivity was found as protocatechuic and vanillic acids, but 3 / of the dose was shown to be dehydroxylated to m-and p-hydroxybenzoic acids and 2 /ₒ to m-methoxybenzoic acid.

Our results confirm the pattern of protocatechuic acid metabolism as outlined above, although the minor metabolites formed by dehydroxylation were not observed on the chromatograms. A new finding is the presence of considerable quantities of catechol in the acid-hydrolysed urines of rats given protocatechuic acid by the oral but not by the intraperitoneal route. This indicates that the catechol is of intestinal origin, which is substantiated by the *in vitro* results showing that extracts of caecal or colon contents or faeces readily decarboxylated protocatechuic acid to catechol under anaerobic conditions This reaction was reduced or abolished when the rats or *in vitro* samples were treated with antibiotics. The finding that intraperitoneal administration did not give rise to urinary catechol suggests that protocatechuic acid does not undergo appreciable excretion in the bile, and this was confirmed in a biliary study showing that only small amounts were excreted by this route. However vanillic acid was readily detected in the acid-hydrolysed bile samples, and this is in agreement with the results of DACRE & WILLIAMS (1962), who found that

the bile contained 13 / of the ^{14}C after intraperitoneal injection of 100 mg/kg of protocatechuic acid and that 70 / of this biliary ^{14}C was a glucuronide of vanillic acid.

It is also pertinent to view the results of DACRE & WILLIAMS (1962) in the light of the decarboxylation reaction They found that the urinary excretion of ^{14}C was increased from 70 / to 86 / when the rats were pretreated with neomycin to destroy intestinal bacteria before dosing with protocatechuic acid. As the ^{14}C was located in the carboxy group of the protocatechuic acid used in their studies, decarboxylation would produce nonradioactive catechol, and the ^{14}C would presumably be lost as respiratory carbon dioxide. Inhibition of the intestinal microflora by the antibiotic treatment would reduce catechol formation and thereby increase the recovery of urinary radioactivity

Small amounts of catechol were seen on the chromatograms of the acid-hydrolysed urines of untreated rats. Catechol has previously been shown to be among the phenolic compounds normally found in urine from rabbits, cattle and man (WILLIAMS 1959 p 293 VON EULER & LISHAJKO 1959) BOOTH *et al* (1960) have postulated that urinary catechol arises from quinic acid (1 3 4 5-tetrahydroxycyclohexane carboxylic acid) and shikimic acid (3 4 5-trihydroxy 1-cyclohexene 1-carboxylic acid), because it was found in the urine after but not before, administering these compounds to rats maintained on a purified diet. They also detected vanillic acid in the hydrolysed urines. BOOTH & WILLIAMS (1963b) reported that extracts of rat caecal contents and faeces convert quinic acid to catechol. These results suggest that quinic and shikimic acids may be converted in the intestine to protocatechuic acid, which can be partly decarboxylated to catechol and partly absorbed and then be methylated to vanillic acid. It seems reasonable to assume that protocatechuic acid itself is also responsible for urinary catechol, as it has been found present in almost every plant systematical examined (HARBORNE & SIMMONDS 1964) and is therefore a normal dietary component for man and many animals.

The metabolism of gallic acid after oral or intraperitoneal administration to the rat and rabbit has been studied by BOOTH *et al* (1959) who found the major urinary metabolite, besides gallic acid itself to be 3,5-dihydroxy-4-methoxybenzoic acid. WATANABE & OSHIMA (1965) administered gallic acid orally to rabbits at a dose level of 500 mg/kg and detected gallic acid, 3,5-dihydroxy-4-methoxybenzoic acid and pyrogallol in the urine. Our results confirm these findings and indicate also that the decarboxylated compounds, pyrogallol and 2-O-methylpyrogallol, are major urinary metabolites when gallic acid is given to rats orally but not intraperitoneally These findings differ from those of BOOTH *et al* (

who found small amounts of 2-O-methylpyrogallol in the urine of rats after oral dosage of gallic acid and both pyrogallol and 2-O-methylpyrogallol in the urine after intraperitoneal dosage The latter difference in the handling of gallic acid may be related to the fact that they used a higher dose level (100 mg per rat compared with 32–34 mg in our studies). However they detected both of the decarboxylated metabolites in the urine of rabbits fed on a diet containing gallic acid.

Intestinal decarboxylation of gallic acid and its metabolites is not entirely precluded when the compound is given by the intraperitoneal route, because of possible biliary excretion. Our biliary studies indicate that gallic acid can be excreted in the bile to a small extent, since a trace was detected after hydrolysis of the biles of one rat after oral and one rat after intraperitoneal dosage The main biliary metabolite of gallic acid is a conjugate of 3,5-dihydroxy-4-methoxybenzoic acid, and to this extent its metabolism is similar to that of protocatechuic acid, which is excreted in the bile mainly as a conjugate of vanillic acid

Booth *et al* (1959) found no evidence for the presence of pyrogallol or its metabolites in the urine after its oral dosage, although they subsequently reported the presence of pyrogallol conjugates and a trace of 2-O-methylpyrogallol in rat urine after administration of pyrogallol (Masri *et al* 1962) Our results show that pyrogallol, after oral or intraperitoneal dosage, is excreted in the urine as conjugates of itself and 2-O-methylpyrogallol, for the phenols are readily observed on chromatograms of the extracts of hydrolysed urines

It is possible that the 3,5-dihydroxy-4-methoxygallic acid excreted in the bile after gallic acid dosage may be decarboxylated in the intestine and thereby give rise to urinary 2-O-methylpyrogallol. However the amount in the bile is too small to account for the observed levels of urinary 2-O-methylpyrogallol after administering gallic acid, and it is clear that methylation of some of the pyrogallol absorbed after its formation in the intestine is the principal source of 2-O-methylpyrogallol.

The presence of pyrogallol in normal human urine has been reported by Tompsett (1958), who stated that it is probably derived from gallic acid by decarboxylation in the alimentary tract. Both the *in vivo* and *in vitro* results of our investigation support this assumption. The decarboxylation of benzoic acid derivatives by the intestinal flora therefore seems to be of some importance in the handling of these compounds by the body It remains to be seen which compounds are so affected, but salicylic acid (2-hydroxybenzoic acid) and γ-resorcylic acid (2,6-dihydroxybenzoic acid) are not decarboxylated by rat faeces extracts (Scheline, unpublished)

The presence of resorcinol in the urine of some human subjects as a monosulphate ester has been reported by CURZON (1957) He found that chromatograms of approximately 23 / of 265 urines tested showed a trace and 2–3 / a clear to strong spot due to this substance A later report (CURZON & PRATT 1964) showed that the resorcinol arose from the action of intestinal bacteria on substances present in tea, probably m-dihydroxy compounds. Our results show that gallic acid was readily decarboxylated to pyrogallol, which was then dehydroxylated to resorcinol when incubated with rat faecal extracts. When caecal or colon contents were used, however the latter reaction was greatly reduced, and only traces of resorcinol were found. The results from the studies of gallic acid metabolism show however that small amounts of resorcinol are often detected in the hydrolysed urine samples. This suggests that gallic acid, which is also found in tea, may be a precursor of urinary resorcinol in some animals or in human subjects having an intestinal microflora containing appropriate bacteria.

Although no great emphasis has hitherto been placed on the effect of the intestinal microflora on the metabolism of organic compounds in animals, it is clearly a factor to be considered. Oral dosage of slowly or poorly absorbed substances or biliary excretion of a compound or its metabolites may result in their metabolism by the intestinal microorganisms and thereby affect the pattern of excretion. Some types of metabolic reaction can be carried out both in the tissues of animals and in the intestine These include the reduction of nitro groups (WILLIAMS 1959 p 410 GLAZKO *et al* 1952), the reduction of azo compounds (FOUTS *et al* 1957 RADOMSKI & MELLINGER 1962 JONES *et al* 1964 SCHELINE & LONGBERG 1965) and the hydrolysis of amides (WILLIAMS 1959 p 156 p.509) Other reactions, such as the removal of aromatic hydroxy groups (DEEDS *et al* 1957 SHAW *et al* 1961 BOOTH & WILLIAMS 1963a & b KAIHARA & PRICE 1963) and the decarboxylation of phenolic acids, appear to be confined to the intestinal microflora. The hydrolysis of glucuronides *also belongs largely to the latter category and may be an important feature* in the metabolism of those compounds excreted in the bile as glucuronide conjugates. When glucuronides are given by injection, however they are excreted essentially unchanged, as they apparently are unable to penetrate into the tissues where β-glucuronidase is located (WILLIAMS 1959 p. 291) From these considerations it appears that the effect of the intestinal microflora on the metabolism of organic compounds in animals is of a degradative rather than a synthetic nature.

Summary

Protocatechuic acid (3 4-dihydroxybenzoic acid) and gallic acid (3 4, 5-trihydroxybenzoic acid) after oral or intraperitoneal administration to rats, were excreted in the urine unchanged and as methylated derivatives (vanillic acid (4-hydroxy 3-methoxybenzoic acid) and 3,5-dihydroxy-4-methoxybenzoic acid, respectively) and as acid-labile conjugates of these compounds.

Decarboxylation of protocatechuic acid to catechol and of gallic acid to pyrogallol was observed when the acids were given by the oral but not by the intraperitoneal route. The decarboxylated compounds were excreted in the urine in a conjugated form Biliary excretion was not a major excretory route, and the metabolites found in the bile were mainly conjugates of the methylated derivatives

Decarboxylation of the phenolic acids readily occurred when they were incubated anaerobically with extracts of caecum or colon contents or faeces. This reaction was inhibited by oxytetracycline and to a lesser extent by neomycin, penicillin and chloramphenicol. When rats were treated with oxytetracycline or neomycin before dosing with protocatechuic or gallic acid to inhibit the intestinal microflora, the urinary excretion of the decarboxylated metabolites was greatly reduced or abolished. Resorcinol was formed from gallic acid or pyrogallol by faecal extracts and to a lesser extent by extracts of intestinal contents. It was also detected in small amounts in the urine of some rats given gallic acid or pyrogallol.

The decarboxylation of dietary components is suggested as a mechanism for the formation of urinary catechol, pyrogallol and resorcinol.

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From the Institute of Pathology Karolinska Sjukhuset and the Institute of Hygiene, Karolinska Institutet, Stockholm, Sweden.

The Effect of Oxolamine Citrate on Experimentally Produced Inflammation in the Respiratory Organs

By

Sven Dahlgren and Tore Dalhamn

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Cigarette smoke and air pollution are among the commonest causes of disorders of the respiratory tract. Sudden increases in air pollution can produce acute symptoms, but this is a rare occurrence compared with the chronic forms of bronchitis and bronchial asthma, emphysema, etc. These diseases are notoriously prevalent in the British Isles, but in recent years they have attracted increasing attention in other countries, particularly in the U S.A In Sweden, chronic bronchitis and allied conditions have hitherto been regarded as relatively rare. However the intensification of air pollution in our cities and the increase in cigarette smoking have focused attention on these problems here too

Chronic productive cough most days for three months or more of each of two successive years is a commonly accepted British definition of chronic bronchitis. In the U.S.A. HEIMAN (1964) has defined chronic bronchitis as "a clinical disorder characterized by excessive mucus secretion in the bronchial tree It is manifested by chronic or recurrent productive cough. Arbitrarily these manifestations should be present on most days for a minimum of three months in the year and for not less than two successive years"

If the source of respiratory irritation cannot be removed, chronic bronchitis tends to be a highly refractory condition. No adequate drug treatment is available as yet. Therefore, when a new drug is shown to possess anti inflammatory properties, its potential usefulness in chronic inflammatory diseases of the respiratory tract offers a promising field for investigation

The pharmacological properties of oxolamine (3-phenyl-5β-diethylaminoethyl-1 2, 4-oxadiazole) were studied in animal experiments by

SILVESTRINI & POZZATTI (1961) The antitussive action of oxolamine was shown to be predominantly peripheral, with a relatively small effect on the cough centre. DALHAMN (1965) has likewise observed an antitussive action of oxolamine citrate in animal experiments.

An anti-inflammatory action of oxolamine citrate was also described by SILVESTRINI & POZZATTI (1961) They tested the ability of oxolamine to diminish oedema produced in the rat foot by the method of RANDALL, SELITTO & VALDES (1957)

Clinical observations on the antitussive and anti-inflammatory action of oxolamine citrate were published by CORBELLA (1960) DEIDDA (1960) REPACI (1960) and TROPIA (1961)

Trials on human subjects, however present many pitfalls as tests of the efficacy of drugs in chronic respiratory tract inflammation. We have therefore tested the anti-inflammatory action of oxolamine citrate on the respiratory organs of guinea pigs

Bronox® was the oxolamine citrate preparation used. Parallel tests were run with a substance of known anti inflammatory properties – phenylbutazone.

Methods

Two series of guinea pigs were used, each containing 20 animals. Each series was subdivided into 4 groups of 5 guinea pigs (A, B, C and D).

The animals in group A received no pharmacological substances, but in all other respects were treated in the same way as the experimental groups.

In groups B, C and D non-bacterial inflammation f the respiratory tract was produced by inhalation of acrolein aerosol (WINTER & PLATAKER 1954). The strength of the acrolein solution was 0.025 %, and the exposure was made for 10 minutes in a plexiglass cylinder measuring bout 200 × 120 mm and fitted with Pari nebuliser (fig. 1).

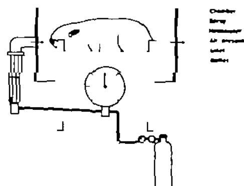


Fig. 1 A Pari nebuliser fitted to plexiglass cylinder used for exposure to acrolein.

Immediately after the acrolein inhalation, intraperitoneal injections were given; they were

Group C, oxolamine citrate, 80 mg/kg body weight
Group D, phenylbutazone, 30 mg/kg body weight
Group A, sodium chloride, 1.5 ml/kg body weight.

The injections were repeated in all three groups after 3, 6, 9, 24, 28, 32, 48, 52, 56, 72 and 76 hours. The repeat doses were 53.3 mg/kg oxolamine citrate, 20 mg/kg phenylbutazone and 0.1 ml/100 g sodium chloride.

The sizes of the doses administered were based on the recommendations for therapeutic dosage.

Phenylbutazone (3,5 dioxy 1,2-diphenyl-4-n-butyl-pyrazolidine, Butazolidin) was used because of its known anti-inflammatory effect (VON RECHENBERG 1961). Moreover, we wished to check the quality of the experimentally produced inflammation by observing its *response to this substance.*

The experiments were stopped after 80 hours. This time was chosen after pilot experiments had shown that the inflammatory changes were then maximally developed. Thirteen of the guinea-pigs died before the 80-hour limit. They were examined immediately after death; the animals that survived at the end of the experiments were killed by bleeding and *immediately autopsied.*

The thoracic organs were removed en bloc and examined macroscopically. The lungs were then dissected free and weighed. The thoracic organs were fixed in 10% formalin. Material for microscopy was taken from the trachea and from the bronchi of both lungs. Tissue from all lung lobes was embedded in paraffin, sectioned and stained with van Gieson *and haematoxylin-eosin.*

The inflammatory changes seen at microscopy – oedema, granulocyte infiltration and hyperaemia – were graded according to the following scale

0 = no inflammatory changes
0.5 = mild inflammation
1.0 = fairly severe inflammation
2.0 = severe inflammation.

The macroscopic and microscopic assessments were made by a pathologist who had no prior knowledge of the groups from which the individual guinea pigs had been taken.

Results

The macroscopic and microscopic observations are reported in tables 1 and 2. The results for the various groups are summarized in table 3.

Of the 10 guinea pigs that received no aerosol inhalation (groups A_1 and A_2) 3 showed mild inflammation of the pulmonary tissue. In the other 7 guinea pigs the structure and air content of the lungs were normal (fig. 2). The bronchi and the trachea were lined with normal ciliated epithelium (fig. 3).

Most of the guinea pigs injected intraperitoneally with NaCl after inhalation of acrolein (groups B_1 and B_2) had macroscopic inflammatory changes in the pulmonary parenchyma. Varying degrees of dense, fairly highly vascularized infiltration of pneumonic appearance were seen in nearly all of these animals. Some of them also showed moderately severe

Table 1

Observations on guinea pigs after i halation of acrolein and intraperitoneal i jection of NaCl oxolamin citrat or phenylbutazone

Group	Treatment	Guinea pig no.	Body weight (g)	Weight of lungs (g)	Relative lung weight (g/100 g)	Microscopy: Inflammatory changes in lungs	Microscopy: Inflammatory changes in bronchi	Microscopy: Inflammatory changes in trachea	Total grading of inflammation
A	None (controls)	1	310	1 90	0.61	0	0	0	0
		2	275	1.55	0.56	0.5	0	0	0.5
		3	270	1 45	0.54	0	0	0	0
		4	255	1 45	0.57	0	0	0	0
		5	210	1.30	0.62	0	0	0	0
B	Acrolein aerosol + sodi m chloride	6	230	2.10	0.91	1	0.5	1	2.5
		7	200	2.25	1 13	0	1	1	2
		8	230	1 65	0.72	1	0.5	0	1.5
		9	310	5 40	1 74	1	1	1	3
		10	300	3.85	1.28	2	1	2	5
C1	Acrolei erosol + olamine citrate	16	240	2.20	0.92	0	0	0	0
		17	290	2.10	0.84	1	1	1	3
		18	280	2.15	0.77	0	0.5	0	0.5
		19	210	1.20	0.57	0	0	0	0
		20	270	2.10	0.78	0	0.5	0.5	1
D1	Acrolein aerosol + phenylbutazone	11	220	1.50	0.68	0	1	0	1
		12	220	1 70	0.77	1	0	0	1
		13	210	2.90	1 19	0	0.5	0	0.5
		14	200	2.00	1.00	1	1	1	3
		15	300	2.10	0.70	1	1	0.5	2.5

0 = no inflammatory changes 0.5 = mild inflammation 1.0 = fairly severe inflammatio 2.0 = severe inflammation

Table 2.

Observations on guinea pigs after inhalation of acrolein and intraperitoneal injection of NaCl oxolamine citrate or phenylbutazone

Group	Treatment	Guinea pig no	Body weight (g)	Weight of lungs (g)	Relative lung weight (g/100 g)	Microscopy Inflammatory changes in lungs	bronchi	trachea	Total grading of inflammation
A_1	None (controls)	26	390	2.15	0.55	0.5	0	0	0.5
		27	360	1.90	0.53	0	0	0	0
		28	460	2.15	0.47	0.5	0	0	0.5
		29	350	1.70	0.49	0	0	0	0
		30	310	1.85	0.60	0	0	0	0
B_1	Acrolein aerosol + sodium chloride	31	300	4.95	1.65	1	1	1	3
		32	370	2.55	0.80	1	1	1	3
		33	300	7.70	2.57		1	1	4
		34	290	2.05	0.71	1	1	0.5	2.5
		35	290	2.05	0.71	1	1	0	2
C	Acrolein aerosol + oxolamine citrate	41	300	2.10	0.70	0	0.5	0.5	1
		42	300	2.55	0.85	0	0.5	1	1.5
		43	60	5.40	2.08	1	1	1	3
		44	290	4.10	1.41	0.5	0.5	0.5	1.5
		45	225	4.50	2.00	1	0.5	0	1.5
D_1	Acrolein aerosol + phenylbutazone.	36	295	5.35	1.82	1	1	0.5	2.5
		37	280	2.25	0.80	0.5	1	0.5	2
		38	290	3.50	1.21	1	1	1	3
		39	270	5.95	2.21	1	1	1	3
		40	260	4.85	1.87	1	1	1	3

0 — no inflammatory changes 0.5 — mild inflammation 1.0 — fairly severe inflammation 2.0 — severe inflammation

Table 3

Means of observations in tables 1 and 2

Group	Treatment	Mean grading of inflammation								Mean relative lung weight	
		Series I (table 1)				Series II (table 2)				Series I (table 1)	Series II (table 2)
		lungs	bronchi	trachea	total	lungs	bronchi	trachea	total		
$A_1 + A_2$	None (controls)	0.1	0	0	0.1	0.2	0	0	0.2	0.58	0.53
$B_1 + B_2$	Acrolein aerosol + sodium chloride	1.0	0.8	1.0	2.8	1.2	1.0	0.7	2.9	1.16	1.29
$C_1 + C_2$	Acrolein aerosol + oxolamine citrate	0.2	0.4	0.3	0.9	0.5	0.6	0.6	1.7	0.78	1.40
$D_1 + D_2$	Acrolein aerosol + phenylbutazone	0.6	0.7	0.3	1.6	0.9	1.0	0.8	2.7	0.87	1.58

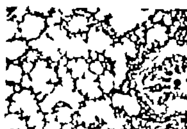


Fig. 2. Normal lung (Animal No. 3).

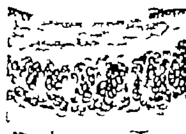


Fig. 3. Normal trachea (Animal No. 4).



Fig. 4. Marked inflammatory changes of the lung with emphysematous blebs and destruction of bronchial epithelium (Animal No. 10).

pulmonary emphysema. No other abnormalities were macroscopically visible in the thoracic organs or elsewhere.

Microscopy showed in the guinea pigs of the B groups that the pulmonary parenchymal changes consisted of agglomerations of inflammatory cells, which were often extensive and contained mainly neutrophil granulocytes (fig. 4). Small areas of bleeding and oedema were found in the most severely affected lungs. The macroscopically observed emphysema was microscopically confirmed (fig. 5).

In both wide and fine bronchi there were varying amounts of granulocytes. The bronchial epithelium was partly shed in the animals with the most severe changes. Sections from the trachea showed essentially the

same picture as in the larger bronchi. The tracheal wall, too was fairly heavily infiltrated with granulocytes, and in places the lumen contained abundant leucocytes (fig. 6).

When inflammatory changes were present, they were as a rule generalized in the respiratory tract, involving pulmonary parenchyma as well as bronchi and trachea

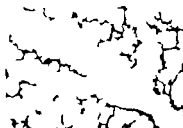


Fig. 5 Emphysema in the animal treated with sodium chloride (Animal N 10).



Fig. 6. Inflammatory granulocytes are noted within the tracheal mucosa and tracheal lumen. The epithelium is destroyed and has loosened from its base (Animal No. 7).



Fig. 7 Trachea from the animal treated with oxolamine citrate. Note the subepithelial hyperaemia. The epithelium is thinner than normal and the cilia have partly disappeared No granulocytic infiltration is noted (Animal No. 19).

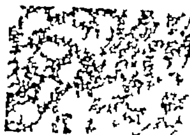


Fig. 8. Hyperaemia of the lung from an animal treated with oxolamine citrate. No inflammatory changes are noted. (Animal No. 18).

Of the 10 guinea pigs injected with oxolamine citrate (groups C_1 and C_2), 8 showed inflammation of the respiratory tract. This was of essentially the same type as in the NaCl-treated groups, but as a rule was considerably less severe. No pathological changes were discernible in 2 animals. Several of the guinea pigs showed varying degrees of hyperaemia in the tracheal wall and in the lungs. The tracheal epithelium in these animals was rather thin and the cilia were partially destroyed. The number of goblet cells seemed to be reduced (figs. 7 and 8).

When phenylbutazone was injected after exposure of the guinea pigs to acrolein (groups D_1 and D_2), they showed inflammatory changes similar to but milder than those of the NaCl-treated groups. In only 1 of the 10 phenylbutazone-treated guinea pigs, however, could the pathological changes in the respiratory organs be classified as mild. Emphysema of varying severity was present besides inflammatory reactions.

The relative weights of the lungs are shown in tables 1 and 2. In all but one of the acrolein-exposed animals the relative lung weight was higher than in the controls. In the first series (table 1) the various subgroups clearly differed in relative lung weights. The highest mean value was found in the NaCl injected guinea pigs. The mean relative lung weight in the oxolamine citrate group (C_1) was significantly lower than that in the NaCl group (analysis of variance) (B_1).

The relative lung weights of the acrolein-treated guinea pigs in the second series (table 2) were often considerably higher than those in the first series. The means of the values in table 2 appeared to be greater in the oxolamine citrate (C_2) and phenylbutazone (D_2) groups than in the NaCl group (B_2). The differences were, however, not statistically significant (analysis of variance).

In both series, oxolamine citrate showed a distinct anti-inflammatory action. As judged from the microscopically assessed degree of inflammation plus the relative weight of the lungs, this affect was highly significant (analysis of variance $P < 0.001$). (Table III).

The anti-inflammatory action of oxolamine citrate was superior to that of phenylbutazone.

The emphysematous changes were difficult to classify On the whole, however the guinea pigs injected with NaCl or phenylbutazone showed fairly extensive emphysema, whereas the guinea pigs that had received oxolamine citrate were almost free from emphysema.

Discussion

Inflammatory changes were produced in the respiratory organs of these guinea pigs by exposure to acrolein aerosol In the second of the two experimental series, the inflammatory reaction seemed generally to be stronger than in the first, probably because of the difficulty experienced in obtaining the same concentration of acrolein aerosol in the both Within each series, however the strength of the aerosol was the same.

By injecting the acrolein-exposed guinea pigs with oxolamine citrate and phenylbutazone, the action of these substances was tested on non-bacterial inflammation of the respiratory organs. Oxolamine citrate was in this way found to be a potent anti inflammatory agent Its effect was superior to that of phenylbutazone.

An antitussive action of oxolamine citrate has previously been demonstrated. This implies some impairment of the natural excretory mechanism in the bronchi. That oxolamine nevertheless proved to be a more powerful anti-inflammatory agent than phenylbutazone lends support to the hypothesis of SILVESTRINI & POZZATTI (1961) that the antitussive effect of oxolamine is dependent mainly on its local anti-inflammatory properties

From our experiments it is not possible to deduce the mechanism of the anti-inflammatory action of oxolamine citrate. The hyperaemia observed in the oxolamine-injected guinea pigs, however may be a factor of some relevance.

One result of the combined anti-inflammatory and antitussive actions of oxolamine citrate seems to be that the pulmonary parenchyma is protected to a significant degree from the development of emphysema.

Our experiments thus seem to confirm observations from earlier clinical and experimental studies on the anti-inflammatory action of oxolamine citrate. The combined antitussive and anti-inflammatory effect should be particularly useful in the important group of respiratory disorders that may be classified as chronic non-bacterial bronchitis, e.g. "smoker's cough" Since such inflammatory irritation frequently is associated with pain, the mild local analgesic action of oxolamine citrate should be an added advantage.

Summary

The effect of oxolamine citrate on experimentally produced respiratory tract inflammation is reported. The inflammatory reactions were produced by exposing the experimental animals to an acrolein aerosol. The studies were performed on two identical series of twenty animals. It has been demonstrated in this experiment that oxolamine citrate has a statistically significant anti-inflammatory effect.

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From the Research Laboratories of AB Astra, Södertälje, Sweden

Uptake of Some Tritiated Sympathomimetic Amines by Mouse Brain Cortex Slices In Vitro

By

S. B. Ross and A. L. Renyi

(Received March 28, 1966)

Tritiated noradrenaline (Dengler, Spiegel & Titus 1961a & b Dengler *et al* 1962 Ross & Renyi 1964) and metaraminol (Ross & Renyi 1966a) are actively taken up by brain cortex tissues *in vitro* The uptake of noradrenaline is inhibited by other sympathomimetic amines (Ross & Renyi 1964) In the present study we have investigated the uptake of some of these amines by brain tissue *in vitro* *viz* dopamine, isoprenaline, tyramine and amphetamine.

Methods

The tritiated amines were incubated with the cortex tissue from mouse brain as described previously (Ross & Renyi 1964). The incubation mixture contained bout 100 mg of brain slices, 0.2 mμmol of the tritiated amine under investigation, 2 ml of Krebs-Henseleit solution and the inhibitor to be tested. The incubation tmosphere was 93.5 % O_2 and 6.5 % CO_2. When short ncubation times were used, the slices were pre-incubated at 37 for five minutes before the addition of the amine. The tissues were extracted with ethanol and the radioactivities recorded in liquid scintillation system as previously described (Ross & Renyi 1964).

The radioactivity n the tissue was taken as measure of the amount of the amine taken up by the tissue. This amount was expressed as mμmol of the amine per gram tissue (wet weight) In some cases, the uptak was expressed as the ratio of the concentration of the radioactivity in the slices to that in the incubation solution and the percentage inhibition of the uptak ccording to the formula previously used (Ross & Renyi 1964).

The tritiated amines used were obtained from the New England Nuclear Corp. and the specific activities used were (±)-noradrenaline-7-^{3}H hydrochloride 2.5 per mmol 3,4-dihydroxyphenylethyl-2-^{3}H amine (dopamine) hydrochloride 1.84 per mmol (±)-isoprenaline-7-^{3}H hydrochloride 3.80 per mmol p-hydro yphenylethylamine-^{3}H (tyramine) hydrochloride, generally labelled, 1 73 per mmol (+)-amphetamine sulphate-^{3}H generally labelled, 1.82 c per mmol.

Results

The time curves of the uptake of the amines were recorded in the absence and in the presence of some inhibitors of the uptake (Fig. 1 a & b). Isoprenaline did not accumulate in the tissues above its concentration in the incubation medium (Fig. 1 a) The amount of the radioactivity taken up in the slices after ten minutes incubation was not changed ($p > 0.05$) in the presence of 20 μg/ml of ouabain. The pre-treatment of the animals with the monoamine oxidase (MAO) inhibitor pheniprazine

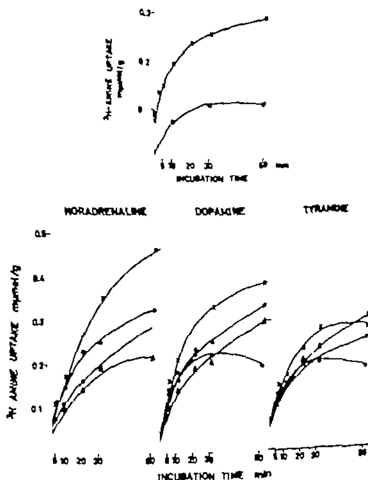


Fig. 1 Time curves of the uptake of tritiated sympathomimetic amines by cerebral cortex slices of mice pre-treated with 10 mg/kg p f pheniprazine on the day before the experiment
a. (+)-Amphetamine × (±)-Isoprenaline ○ b. (±)-Noradrenaline, dopamine and tyramine without × and with inhibitors 2 μg/ml cocaine hydrochloride Δ, 2 μg/ml desmethylimipramine hydrochloride ● 0.03 μg/ml reserpine ○.

Table 1

Effect of phenipradine pre-treatment of the animals on the uptake of tritiated (±)-isoprenaline by cortex slices from mouse brain *in vitro*

Ten mg/kg of phenipradine hydrochloride were injected four hours before the experiment. The incubation was performed for 10 and 60 minutes. The values are means and standard error of mean (s.e.m.) for three determinations.

Treatment	Amounts of isoprenaline in the slices mμmol/g ± s.e.m. 10 min.	60 min.
—	0.071 ± 0.003	0.102 ± 0.002
Phenipradine.	0.083 ± 0.001	0.118 ± 0.003[1]

[1] 0.05 > p > 0.01 (Student's t-test).

increased the amount of the amine in the tissue only to a small extent (Table 1)

(+)-Amphetamine was rapidly accumulated in the slices (Fig. 1a) but the concentration ratio of the amine in the tissue to that in the incubation solution was only slightly diminished with a hundredfold increase in the concentration of the amine in the incubation solution (Fig. 2) in contrast to that found for noradrenaline (DENGLER, SPIEGEL & TITUS 1961a ROSS & RENYI 1964) and for metaraminol (ROSS & RENYI 1966a) The uptake of (+)-amphetamine was not inhibited ($p > 0.05$) by cocaine

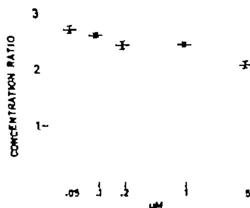


Fig. 2. Accumulation of (+)-amphetamine in cortex slices from mouse brain *in vitro* at different concentrations of amphetamine. Abscissa amphetamine concentration in incubation solution (μM). Ordinate ratio tissue/incubation solution concentration of amphetamine. The incubation time was 45 minutes. The vertical bars indicate standard error of mean for four determinations.

Table 2

Effect of pheniprazine pre-treatment of the animals on the uptake of tritiated tyramine by cortex slices from mouse brain *in vitro*.

Ten mg/kg of pheniprazine hydrochloride were injected on the day before the experiment. The incubation was performed for 5 and 45 minutes. The values are means and standard error of mean (s.e.m.).

Treatment	Amounts of tyramine in the slices mµmol/g ± s.e.m. 5 min.	45 min.
—	0.144 ± 0.007 (n = 4)	0.175 ± 0.003 (n = 4)
Pheniprazine	0.159 ± 0.006[1] (n = 13)	0.270 ± 0.016[1] (n = 4)

[1] $0.01 > p > 0.001$

hydrochloride (20 µg/ml) desmethylimipramine hydrochloride (DMI) (20 µg/ml) ouabain (20 µg/ml) or reserpine (0.03 µg/ml).

Tyramine was also rapidly taken up by the tissues (Fig. 1b) and this uptake was considerably increased after pre treatment of the animals with a MAO inhibitor (Table 2) With a short incubation time there was, however no significant difference between the uptake by the tissues from pheniprazine pretreated mice and untreated animals (Table 2). The uptake of the tyramine was partly inhibited by cocaine, DMI, ouabain and reserpine (Fig. 1b and Table 3), compounds known to inhibit the uptake of noradrenaline at the neurone membrane or the storage of the noradrenaline in the neurone (HILLARP & MALMFORS 1964 LINDMAR & MUSCHOLL 1964 MALMFORS 1965)

Since the enzymatic transformation of tyramine to octopamine may be of importance in the accumulation of the tyramine in the slices, we studied the effect on this uptake of a dopamine-β-oxidase inhibitor sodium diethyldithiocarbamate (GREEN 1964) It had, however no significant ($p > 0.05$) effect at a concentration of 10^{-3} M.

Dopamine was taken up by the slices in the same way as noradrenaline (Fig. 1b) but the initial uptake was somewhat greater for dopamine. Accumulation of dopamine was partly inhibited by cocaine, DMI, ouabain and reserpine (Fig. 1b and Table 4). The uptake of dopamine was somewhat increased after pheniprazine pre treatment of the animals when the incubation period was half an hour or more, but there was hardly any effect with shorter incubation periods.

The inhibitory effect of cocaine at different concentrations on the uptake of tyramine, dopamine and noradrenaline was studied. Incubation

Table 3

Inhibition of the uptake of tyramine by cortex slices from mouse brain *in vitro*
The animals were pre-treated with 10 mg/kg of phenipra zine hydrochlorid on the day before the experiments. The incubation time was 45 minutes. The uptak of tyramine was expressed as the ratio of the concentration of the amine in the slices t that in the medium. The figures are means and standard error of mea for f ur determinations.

Inhibitor	Conc. µg/ml	Concentration ratio ± s.e.m.	Inhibition of uptak %
—	–	2.0 0.2	–
Cocaine HCl	20	1.4 ± 0.08[1])	60
—		2.3 ± 0.1	
Cocaine HCl	2	2.1 ± 0.1	15
—	–	2.0 ± 0.2	
Desmethylimipramine HCl	20	1.6 ± 0.04	40
—		2.3 0.1	
Desmethylimipramine HCl		2.2 ± 0.08	8
—		2.2 ± 0.1	
Ouabain	20	1.3 ± 0.02[3])	75
Ouabain	2	1.8 ± 0.05[2])	33
—	–	2.2 0.1	
Guanethidine	10	1.6 ± 0.07[2])	50
Guanethidine	1	2.0 ± 0.1	17
—		2.3 ± 0.1	–
Reserpine	0.03	1.7 ± 0.05[2])	46

[1]) $0.05 > p > 0.01$ [2]) $0.01 > p > 0.001$ [3]) $p < 0.001$

Table 4

Inhibition of the uptake of dopamine by cortex slices from mouse brain *in vitro*
The incubation time was 30 minutes. The uptake of dopamine was expressed as the ratio of the concentration of the amin in the slices to that in the solution. The figures are means nd standard error of mean for four determinations.

Inhibitor	Conc µg/ml	Concentration ratio ± s.e.m.	Inhibition of uptake %
—		3.3 0.14	
Cocaine HCl	2	1.9 ± 0.05[2])	61
Desmethylimipramine HCl	2	2.2 ± 0.17)	48
Ouabain	20	1.6 ± 0.07[3])	74
Guanethidine	2	2.4 0.10[2])	39
—		3.1 ± 0.08	
Reserpine	0.03	1.7 ± 0.04[3])	67
Tetrabenazine HCl	2	1.8 ± 0.13[3])	62

) $0.01 > 0.001$) $p < 0.001$



Fig. 3. Inhibitory effect of cocaine on the uptake of amines by cortex slices from mouse brain in vitro

The incubation time was 5 minutes with 5 minutes pre-incubation with cocaine before the addition of the amine studied. Each value is the difference between the means of four determinations without and with cocaine. Tyramine △ dopamine ○ noradrenaline ×

periods of five minutes were used in these experiments. Figure 3 shows that the total amount of the cocaine sensitive part of the uptake of the amines was the same for dopamine and noradrenaline but was less for tyramine. Fifty per cent inhibition of the total cocaine sensitive uptake was however obtained at the cocaine concentration of 10^{-6} M for all three amines.

The cocaine sensitive part of the uptake of the amines was studied with different concentrations of the amines in the incubation solution. The amounts of the amines taken up by the slices during the five minutes

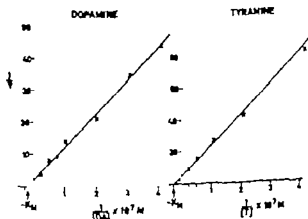


Fig. 4. Lineweaver Burk plot of the cocaine-sensitive part of the uptake of dopamine and tyramine.

The incubation time was 5 minutes with 5 minutes pre-incubation before the addition of the amine. The individual values are the difference between the mean of four determinations without cocaine and four with 30 μM cocaine for each concentration of the amine studied is expressed as mμmol/g/5 min

incubation were approximated as the initial uptake velocities. The data obtained were plotted by the method of LINEWEAVER & BURK (1934) The K_M values for dopamine and tyramine were estimated to be 7×10^{-7} and 4×10^{-7} M, respectively and the V_{max} to be 0.11 and 0.04 mμmol/g/min., respectively (Fig. 4). The corresponding values for noradrenaline obtained from the experiments in a previous investigation (ROSS & RENYI 1964) were 4×10^{-7} M and 0.11 mμmol/g/min. respectively

In contrast to cocaine, reserpine had hardly any inhibitory effect on the uptake of noradrenaline by the slices after a short period of incubation (Fig. 5 and Table 5) but the effect of reserpine increased with increasing

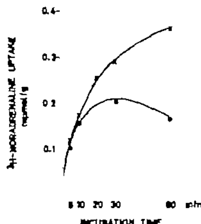


Fig. 5. Inhibition of the uptake of noradrenaline by cortex slices from mouse brain with reserpine *in vitro*
Control × 0.03 μg/ml reserpine O

Table 5

Effect of reserpine pre-treatment of the animals on the uptake of tritiated noradrenaline by cortex slices from mouse brain *in vitro*. Five mg/kg of reserpine were injected intraperitoneally 20 hours before the experiment. The incubation was performed for 5 minutes with 5 minutes pre-incubation before the addition of noradrenaline. The figures are means and standard error of mean for three determinations

Treatment	Amounts of noradrenaline in the slices mμmol/g ± s.e.m.
—	0.111 ± 0.002
Reserpine	0.104 ± 0.006[1])

[1]) $p > 0.05$

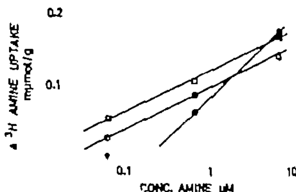


Fig. 6. Inhibitory effect of noradrenaline on the uptake of tritiated dopamine and that of dopamine on the uptake of tritiated noradrenaline by brain cortex slices *in vitro*. The incubation time was 30 minutes. The values are the difference between the mean of four determinations without the inhibiting amine studied and four determinations with the amine at different concentrations.
Noradrenaline on the uptake of dopamine □ Dopamine on the uptake of noradrenaline O.
The filled symbols denote slices from pheniprazine pre-treated animals, 10 mg/kg i.p. given on the day before the experiment.

incubation time. Reserpine seemed to have a somewhat greater effect on the accumulation of dopamine and tyramine than on that of noradrenaline, when the animals were pre-treated with pheniprazine (Fig. 1*b*).

We have previously shown that the uptake of noradrenaline is inhibited by other sympathomimetic amines (Ross & Renyi 1964). Figure 6 shows that noradrenaline inhibited the uptake of tritiated dopamine and *vice versa*. The same results were obtained with tyramine and noradrenaline (Fig. 7). Dopamine and tyramine inhibited the uptake of noradrenaline with steeper dose response curves as compared with the effect of noradrenaline on the uptake of these amines. Pheniprazine pre-treatment of the animals did not change the slope of the dose response curves for the effect of tyramine on the noradrenaline uptake. The slope of the dose response curve of the effect of dopamine on the noradrenaline uptake was shifted, however, so that it was parallel to that of noradrenaline on the dopamine uptake after pre-treatment of the animals with pheniprazine.

Discussion

Recent studies (Hillarp & Malmfors 1964, Lindmar & Muscholl 1964, Carlsson & Waldeck 1965, Malmfors 1965) have shown that cocaine and DMI inhibit the noradrenaline uptake at the neurone membrane transfer sites (the "cell membrane pump") while reserpine destroys

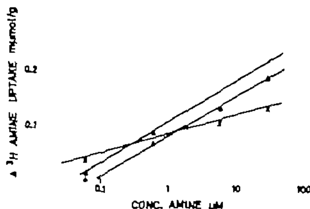


Fig. 7 Inhibitory effect of noradrenaline on the uptake of tritiated tyramine and that of tyramine on the uptake of noradrenaline by brain cortex slices *in vitro*
The incubation time was 45 minutes. The values are obtained as described in the legend to Fig. 6.
Noradrenaline on the uptake of tyramine × Tyramine on the uptake of noradrenaline Δ
The unfilled symbols denote slices from animals pre-treated with phenoprazine, 10 mg/kg i.p. given on the day before the experiment.

the interneuronal storage sites for noradrenaline. The results obtained in the present investigation are in good agreement with these findings.

The *in vitro* accumulation of the amines in the tissue seems to be partly dependent on an unspecific uptake of the amines which to some extent may be an *in vitro* artefact, and partly on a specific, cocaine sensitive active uptake reaction. The relative effectivity of these two uptake mechanisms varied from amine to amine The unspecific uptake was diminished with decreasing lipid solubility of the amine, indicating that this part of the uptake is mainly a result of physicochemical properties of the amine. The active uptake of the amines as defined above may be dependent on the chemical structure of the amine. Although too few amines have so far been studied, some assumptions concerning relationships between the structure and activity can be suggested. The amines with two hydroxyls on the benzene nucleus may have higher activity than compounds with only one. The hydroxyl on the β-carbon seems to have no or little importance for the active uptake of the amines by the tissue studied. The amines without any hydroxyl group may not be taken up by this mechanism. A large substitution on the nitrogen may also abolish the uptake, since isoprenaline was not found to be taken up, in agreement with the previous suggestion based on its slight inhibitory effect on the uptake of noradrenaline (Ross 1963) and with the findings of Hertting (1964) and Andén *et al* (1964)

The transformation of tyramine to octopamine does not seem to be

of any importance for the accumulation of tyramine in the tissue, since the dopamine-β-oxidase inhibitor sodium diethyldithiocarbamate (GREEN 1964) had no effect on the uptake of this amine by the slices.

The "cell membrane pump" inhibitors, cocaine and DMI, had a greater effect than the storage blocking agent reserpine when a short incubation time was used. This finding indicates that the uptake by the membrane is the initially dominant factor. During this time storage mechanism plays a minor role. Prolongation of the incubation time led to an increased effect of reserpine which may be explained by an increasing importance of the storage mechanism. The same explanation may be given for the finding that the uptake of tyramine is initially not influenced by the intact MAO system of the slices.

Although the possibility cannot be excluded that the binding of the amines by the intraneuronal storage sites may have some significance for the initial uptake of the amines by the slices, it seems probable that the results of the kinetic experiments of the cocaine sensitive uptake reflect mainly the "cell membrane pump". According to these experiments tyramine has the same affinity for the transfer sites as noradrenaline and dopamine, but is taken up at a slower rate than the other two amines.

The indirectly acting sympathomimetic amines, e.g. tyramine and amphetamine, are believed to act by releasing noradrenaline from a "labile" store (STJÄRNE 1961; TRENDELENBURG 1961; TRENDELENBURG & CROUT 1964). This pool of noradrenaline which can be released by tyramine seems to be located extracellularly (TRENDELENBURG 1965; FISCHER, KOPIN & AXELROD 1965) near the receptors (FURCHGOTT *et al.* 1963). It is small since only 1 / of the normal noradrenaline content is necessary to restore the response of tyramine in reserpinized animals (CROUT, MUSKUS & TRENDELENBURG 1962). Reserpine can thus abolish the effect of the indirectly acting amines (CARLSSON *et al.* 1957) but noradrenaline can restore the effect (BURN & RAND 1958). Cocaine antagonizes the sympathomimetic effect of these amines (TAINTER & CHANG 1927; BURN & RAND 1958) but potentiates the effects of the catecholamines (FRÖHLICH & LOEWI 1910). All these findings may be understood, if one assumes that the pool of noradrenaline which can be released by tyramine may be identical with those noradrenaline molecules bound to the transfer sites of the neurone membrane (cf. FURCHGOTT *et al.* 1963). The present investigation shows that tyramine has about the same affinity for these sites as noradrenaline and judging from its inhibitory effect on the noradrenaline uptake, amphetamine also has a great affinity for these sites (DENGLER, SPIEGEL & TITUS 1961 b; ROSS & RENYI 1964; IVERSEN 1964). The transfer sites are probably located near the receptors, since the recapture and uptake of noradrenaline seems to be most important way of inactivating

this amine (AXELROD WEIL MALHERBE & TOMCHICK 1959 MUSCHOLL 1960). The amount of noradrenaline molecules at the transfer sites is difficult to estimate but since the uptake mechanism seems to have a great capacity and the whole membrane of the neurone can take up the amine (HILLARP & MALMFORS 1964), the number of molecules bound to these sites may be high enough to account for the effect of the indirectly acting amines. Reserpine does not seem to have any effect on the uptake mechanism at the cell membrane (HILLARP & MALMFORS 1964 LINDMAR & MUSCHOLL 1964 ROSS & RENYI 1966b), but the general depletion of noradrenaline from the tissues may also cause a marked decrease of noradrenaline at the transfer sites. Since cocaine has a great affinity for the transfer sites as judged by its inhibitory effect on the uptake of noradrenaline, this compound may inhibit the effect of the indirectly acting amines by occupying these sites and preventing the release of noradrenaline.

Cocaine releases small amounts of noradrenaline from tissues (HIGUCHI, MATSUO & SHIMAMOTO 1962) which may be associated with its binding to the noradrenaline transfer sites discussed above. With DMI, on the other hand, no release of noradrenaline has been observed (MATUSSEK, RÜTHER & TITUS 1964) However a compound which is bound to these sites without releasing noradrenaline but blocks the noradrenaline releasing effect of tyramine according to the hypothesis discussed above, is quite conceivable.

It may finally be pointed out that tyramine in addition to releasing noradrenaline from the extraneuronal store discussed here, can also release it from the larger intraneuronal pools (NEFF *et al* 1965).

Summary

The uptake of tritiated (±)-noradrenaline, dopamine, tyramine, (+)-amphetamine and (±)-isoprenaline by cortex slices from mouse brain *in vitro* were studied

(+)-Amphetamine accumulated in the tissues but this uptake seems to be an unspecific uptake, since compounds known to inhibit the active uptake and the storage of noradrenaline, *e.g.* cocaine, desmethylimipramine (DMI), ouabain and reserpine, had no effect on the uptake of amphetamine.

(±)-Isoprenaline was not or only slightly accumulated in the tissues.

Tyramine was accumulated in the slices and the amount accumulated after long incubation was considerably increased by pretreatment of the animals with a monoamine oxidase inhibitor The uptake of tyramine was partly inhibited by cocaine, DMI ouabain and reserpine.

Dopamine was taken up to a similar extent to noradrenaline and the inhibitors mentioned above also partly inhibited the uptake of these amines.

The hypothesis is suggested that the noradrenaline molecules bound on the transfer sites of the cell membrane constitute the small "labile" pool of noradrenaline which is released by the indirectly acting amines, *e.g.* tyramine and amphetamine.

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From the Department of Pharmacology Leo Pharmaceutical Products, Ballerup, Denmark

Interaction of Amphetamine With Anticonvulsant Drugs. II. Effect of Amphetamine on the Absorption of Anticonvulsant Drugs

By

H.-H. Frey and Elsemarie Kampmann

(Received March 17 1966)

In a recent paper (FREY 1964) the effect of amphetamine on the activity of anticonvulsant drugs was studied in mice. The finding that amphetamine given orally one hour before the electroshock was without effect on the ED50s of phenobarbital (phenemalum NFN) or diphenylhydantoin, given orally two hours before electroshock, was in agreement with clinical experience. However STILLE (1953) giving amphetamine as well as diphenylhydantoin or phenobarbital by subcutaneous injection one hour before electroshock, had found the ED50s for the two drugs about halved after amphetamine pretreatment. KLISSIUNIS et al (1965) reported recently that the enteral absorption of phenobarbital by rats could be enhanced by simultaneous application of amphetamine. Thus it seemed possible that amphetamine could advance the initiation of anticonvulsant activity with the result that the maximal anticonvulsant effect – usually present 2–3 hours after oral application of phenobarbital or diphenylhydantoin – would be achieved after one hour Should this prove to be so it could explain the results of STILLE as well as our own.

We decided therefore to vary the time interval between the application of phenobarbital or diphenylhydantoin and the testing of anticonvulsant activity in the maximal electroshock seizure test and to re-study the effect of amphetamine pretreatment on the ED50s of the anticonvulsants. In addition, the serum concentrations of the anticonvulsants at the time of testing for anticonvulsant activity were determined.

Methods

All experiments were mice, irrespective of sex, f the Leo strain, weighing 20–24 g. DL-amphetamine sulphate in doses of 3, 5 or 8 mg/kg was always given one hour bef re termination of the experiment (electroshock or decapitation or both), phenobarbital was given 1 and 2 hours, diphenylhydantoin 1–3 hours before this. The total volume of the oral medication never exceeded 10 ml/kg

The maximal electroshock seizure test was pplied as described in our recent paper (FREY 1964). The ED 50s were determined by the method of LITCHFIELD & WILCOXON (1949).

Phenobarbital, diphenylhydantoin or ethosuximide was extracted from the pooled serum of 6 mice and determined spectrophotometrically by the methods used in a recent study (FREY & KAMPMANN 1965) At least 6 determinations were made for each point.

Results

1 *Phenobarbital*

One hour after oral application the ED50 of phenobarbital in the maximal electroshock seizure test was about twice as high as two hours after application (table 1). It can also be seen from the table that simul taneous application of amphetamine gave rise to a considerable increase in the dose necessary to protect 50 / of the mice against electroconvul sions. The rise in the ED50 was paralleled by a decrease in the serum concentrations of phenobarbital. This decrease was most pronounced

Table 1

Anticonvulsant activity and serum concentrations of phenobarbital without or along with amphetamine. Amphetamine wa always given 1 hour before either testing the anticonvulsant activity in the maximal electroshock seizure test or beheading the mice.

Amphetamine	1h ED50	Serum concentratio µg/ml			2h ED50[1]	Serum concentration µg/ml	
		20 mg/kg	30 mg/kg	40 mg/kg		20 mg/kg	30 mg/kg
	24 mg/kg (20–30)	20 ± 3 6	30 ± 3	43 ± 1.8	13 mg/kg (12–15)	23 ± 2.9	32 ± 1 7
3 mg/kg	42 mg/kg (36–48) p < .01	20 ± 3.6	26 ± 5.3	29 ± 7 1 p < 02	14 mg/kg (12 16)	21 ± 4.1	30 ± 3 4
5 mg/kg	73 mg/kg (49–110) p < .001	18 ± 2.9	21 ± 0.6 p < .01	29 ± 2.9 p < .01	15 mg/kg (13–18)	23 ± 2.4	35 ± 5.8
8 mg/kg	73 mg/kg (49–110) p < .001	15 ± 1.2 p < .05	18 ± 2 4 p < .01	26 ± 7 1 p < .01	11 mg/kg (9.3–13)	22 ± 1.2	34 3.5

[1]) T ken from FREY (1964).

Table 2

Anticonvulsant activity and serum concentrations of diphenylhydantoin without or along with amphetamine. Amphetamine was always given 1 hour before either testing the anticonvulsant activity in the maximal electroshock seizure test or beheading the mice

Amphetamine	1h ED50	Serum-conc. µg/ml 20 mg/kg	2h ED50¹)	Serum-conc. µg/ml 20 mg/kg	3h ED50	Serum-conc. µg/ml 20 mg/kg
–	24 mg/kg (19–30)	16.5 ± 3 9	14 mg/kg (12–16)	18.5 ± 1.5	15 mg/kg (12 19)	22 ± 4
3 mg/kg	68 mg/kg (46–100) p < 001	12 ± 4.3	19 mg/kg (15.5– 4) p < 05	13 ± 1 7 p < .01	15 mg/kg (13–17)	20 ± .8
5 mg/kg	54 mg/kg (44–66) p < .01	12 ± 5 1	19 mg/kg (15.5–24) p < 05	15 ± 2.2 p < 01	13 mg/kg (11 15)	20 ± 2.1
8 mg/kg	42 mg/kg (36–49) p < 01	9.5 ± 3 4 p < .01	17.3 mg/kg (12.5–24)	9.5 ± 1.5 p < .01	12.5 mg/kg (10–15)	19 ± 2.4

1) Taken from Frey (1964).

after the higher doses of the anticonvulsant, when the serum concentration reached only 60 % that of the control group It should, however be remarked that an increase in the amphetamine dose from 5 to 8 mg/kg, though leading to no further increase in the ED50, led to a further decrease in the serum concentration of phenobarbital.

When the electroshock was applied two hours after phenobarbital and one hour after amphetamine (2h ED50) the latter no longer displayed a significant influence on the anticonvulsant ED50 of the former and the serum concentrations of phenobarbital in groups with and without pretreatment were consistently identical

2. *Diphenylhydantoin*

Table 2 summarises the corresponding results with diphenylhydantoin. Curiously the elevation of the 1h ED50 was most pronounced after the lowest dose of amphetamine and showed a significant decrease after the application of the higher amphetamine doses though remaining far above the ED50 for diphenylhydantoin without amphetamine pretreatment. In spite of this the serum concentration of the anticonvulsant proved to be most decreased after the highest dose of amphetamine. The same trend appears in the 2h ED50s. These were slightly elevated after 3 and 5 mg/kg

amphetamine, and this rise was paralleled by a decrease in serum concentrations. But after 8 mg/kg amphetamine the ED50 was no longer significantly raised, though the serum concentration of diphenylhydantoin roughly corresponded to one half of that in the control group without amphetamine treatment. First when amphetamine is given 2 hours after diphenylhydantoin and one hour before electroshock (3h ED50) neither ED50s nor serum concentrations are altered significantly by the pretreatment. However a slight trend towards lower ED50s and serum concentrations is still apparent.

3. *Ethosuximide*

In the previous study (FREY 1964) an important antagonistic effect of amphetamine could be shown against the anticonvulsant effect of trimethadione and ethosuximide in the pentetrazole seizure threshold test. This effect was then interpreted as a central antagonism but in the light of our present results with phenobarbital and diphenylhydantoin it seems just as likely that it was due to retarded absorption of the anticonvulsant drugs. Therefore, we determined the serum concentrations of ethosuximide one hour after the oral application of the drug alone or along with amphetamine. The results (table 3) show that here also ab-

Table 3

Anticonvulsant activity and serum concentrations of ethosuximide without or along with amphetamine. Both drugs were given orally 1 hour either before testing the anticonvulsant activity in the pentetrazole seizure threshold test or beheading the mice.

Amphetamine	1h ED50[1])	Serum concentration µg/ml 200 mg/kg
–	190 mg/kg (160–230)	161 ± 28
3 mg/kg	300 mg/kg (260–360) $p < .02$	129 ± 36
5 mg/kg	300 mg/kg (260–360) $p < .02$	145 ± 34
8 mg/kg	360 mg/kg (300–420) $p < .01$	101 ± 21

[1]) Taken from FREY (1964).

sorption was retarded by amphetamine, though the difference from the control group only became significant with the highest amphetamine dose. This is partly due to the somewhat large variation in the serum concentration of ethosuximide which in turn is the consequence of the drug's extremely rapid metabolism in the mouse (FREY & KAMPMANN 1965).

Supplementary experiments in which the anticonvulsant effect of ethosuximide in the pentetrazole seizure threshold test was plotted against the serum concentration of the drug one hour after its oral application showed that the lower concentrations reached along with amphetamine may be sufficient to explain the observed antagonism.

Discussion

Our new results necessitate a certain reinterpretation of the results of our previous experiments (FREY 1964). It was then stated that amphetamine was without effect on the anticonvulsant action of phenobarbital and diphenylhydantoin. This statement has now to be modified to indicate that amphetamine is without effect on the maximal anticonvulsant activity that can be obtained by the anticonvulsant drugs mentioned. However, amphetamine medication has been shown able to retard intestinal absorption of the drugs studied and thereby to delay initiation of the anticonvulsant action (phenobarbital and diphenylhydantoin) and with diphenylhydantoin to postpone the time of maximal efficacy. The results in table 1 show that the delay in absorption was most pronounced after the higher doses of the anticonvulsant.

The delay in the absorption of drugs may partly be the result of a delay in gastric emptying and a decrease in intestinal motility that were shown by VAN LIERE *et al.* (1952) and NORTHUP & VAN LIERE (1953) in rat, dog, and man on fairly low doses of amphetamine. Further, the long-acting vasoconstrictor may be able to curtail the circulation in the gastrointestinal tract, thereby retarding the distribution of absorbed drug.

Closer inspection of tables 1 and 2 reveals some results to support the view of an anticonvulsant property of amphetamine itself as held by some authors (for references vide FREY 1964).

1. Though the 1h ED50 for phenobarbital remains the same when the amphetamine dose is raised from 5 to 8 mg/kg, the serum concentrations were always lower after the higher amphetamine dose.

2. With diphenylhydantoin the 1h ED50 was highest when it was used along with the lowest dose of amphetamine; with higher doses of amphetamine both ED50s and serum concentrations decreased significantly.

3. The 2h ED50 for diphenylhydantoin along with 8 mg/kg amphetamine is not significantly higher than in the control experiment with-

out amphetamine, but the serum concentration of the anticonvulsant is only about one half that in the control series

This synergistic effect of amphetamine, however ceases to be significant when the maximal effect of the anticonvulsants is reached (2h ED50 of phenobarbital and 3h ED50 of diphenyldydantoin) The same should apply to clinical therapy when steady concentrations of the anticonvulsant drugs are maintained by an appropriate dose schedule.

With ethosuximide determination of a corresponding end-point was impossible, since this drug is metabolized so rapidly in the mouse that it is almost without anticonvulsant effect only 2 hours after application.

The results of our study stress the importance of chemical determination of the drugs involved in studies of drug synergism or antagonism. Disregard of the chemical equivalent of the measurable pharmacodynamic effects may otherwise lead to misinterpretations of the experimental results.

Summary

The simultaneous use of the anticonvulsant drug phenobarbital, diphenylhydantoin or ethosuximide with amphetamine resulted in a considerable delay in intestinal absorption of the anticonvulsants and an apparent antagonism at least during the period preceding the maximum effect of the drugs. With diphenylhydantoin the point of maximal effect was postponed for about one hour when it was given along with amphetamine.

On the other hand there was some evidence for a synergistic effect of amphetamine during the rise in anticonvulsant effect of phenobarbital and diphenylhydantoin, characterized by unchanged or even falling ED50s in spite of a progressive decrease in the serum concentrations.

The importance of concomitant chemical determinations of the drugs involved in studies on drug synergism or antagonism is stressed.

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From The Central Laboratory Kolonien Filadelfia (Chief C. M. Plum), and The Man and Children Department, Kolonien Filadelfia (Chief E. Kiørboe) Dianalund, Denmark

Disulfiramum (Antabuse ®) as Inhibitor of Phenytoin Metabolism

By

Ole Vendelin Olesen

(Received April 12, 1966)

Disulfiramum (antabuse ®) is best known as an inhibitor of liver aldehyde oxydase (KJELDGAARD 1949 GRAHAM 1951) In addition, when administered in man disulfiram can cause serious side effects, probably due to its action on the metabolic changes of other normally occurring substances (HEATH *et al* 1956)

In the hospital at Dianalund, clinical observation has shown that in several cases, patients treated with phenytoin developed phenytoin intoxication after taking disulfiram (KIØRBOE, personal communication) This might have suggested that a potentiating effect of disulfiram was involved, as animal experiments have shown that the drug potentiates the effect of barbiturates (GRAHAM *et al* 1951) In the cases of intoxication in question, however there was good agreement between the phenytoin concentration found in the serum, and the symptoms of intoxication observed clinically It was therefore unlikely that a potentiating effect occurred. A further possibility however was that disulfiram caused errors in the method of SVENSMARK & KRISTENSEN (1963) used for determining serum phenytoin. The aim of the present investigation, therefore, was to exclude any possible gross errors of analysis, and also to determine whether the cases of intoxication were exceptions, or whether they should be regarded as a common phenomenon when combined disulfiram and phenytoin treatment is given.

Material and Method

Four male patients: A, B, C and D all of whom had been treated with phenytoin for a period of years, volunteered to take 400 mg Antabuse daily over a period. *Patient A* aged 79 years, weight 70 kg, was treated with phenytoin 300 mg, primidone (NFN) 750 mg, carbamazepine (Brit.) 800 mg and thioridazine (NFN) 150 mg daily. *Patient B* aged 34 years, weight 65 kg, was treated with phenytoin 400 mg, primidone (NFN) 750 mg, amitriptyline (NFN) 150 mg and chlorpromazine (NFN) 600 mg daily. *Patient C*, aged 58 years, weight 87 kg, received phenytoin 400 mg and primidone (NFN) 1500 mg. *Patient D* aged 24 years, weight 74 kg, received phenytoin 300 mg and chlorpromazine (NFN) 150 mg daily.

During the period of the investigation, antabuse ®, one tablet of 400 mg, was taken every morning at 7.30, and the daily blood samples were taken at 11.30 A.M. A modification (OLESEN, unpublished) of the method of SVENSMARK & KRISTENSEN (1963) was used to determine the phenobarbitone and phenytoin concentrations in the serum. Independent of these determinations, the phenytoin concentrations in the serum were determined by a special chromatographic method (OLESEN 1965).

Results

Figs. 1a & b show the results of the investigation. The level of the respective phenytoin concentration is first determined before starting the administration of 400 mg disulfiram daily. As early as four hours after the ingestion of the first dose of disulfiram, a significant rise in the serum concentrations was observed. This rise continued more or less regularly and in parallel during the course of treatment (9 days) in all four experimental subjects, and even 3–4 days after withdrawal there was no sign of any fall. The effect of disulfiram was very prolonged, and 14 days after its withdrawal, the phenytoin concentrations were still not back to the starting levels, which would normally have been the case after only 4–8 days, if a comparison had been made with the fall after a dose reduction (TVETEN 1963). Unfortunately, as a result of the patients leaving for Christmas holidays, it was impossible to follow the serum concentrations for a longer period, but experience with other cases of phenytoin-disulfiram intoxication shows that a period of about 3 weeks must be allowed for normal conditions to be re-established. A comparison between the results of the spectrophotometric and the visual method shows certain divergences between the numerical values obtained. This is due to the unreliability of the respective methods used, and to the fact that 3 of the subjects, A, B and C, received treatment with primidone, which can give a very variable error in the spectrophotometric method. The divergences were only small in the case of patient D so that the disulfiram present in the blood probably cannot be considered as causing any significant error in the spectrophotometric method. Patients A and B developed symptoms of mild phenytoin intoxication during the experi-

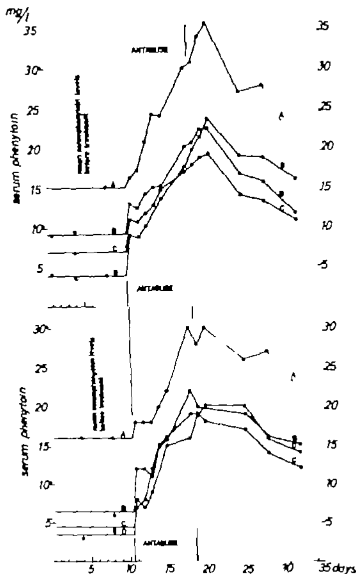


Fig. 1 The serum phenytoin concentrations determined by two different methods (upper part spectrophotometric method, lower part visual method), as a function of time. The serum concentrations were determined in four patients, A, B, C and D who had received treatment with phenytoin over period of years. During the period marked by the vertical lines, 400 mg of disulfiram (antabuse (R)) was administered daily at 7.30 A.M., and blood samples taken at 11.30 A.M.

ment, so that in the case of A it was necessary to withdraw a 24-hour dose of phenytoin between the 29th and 30th days, which is why the curve of elimination is shown stippled between the last two points.

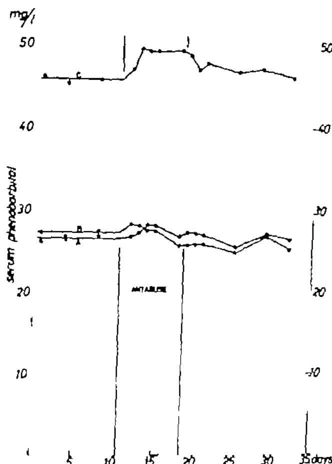


Fig. 2. The serum phenobarbitone concentrations as a function of time. Three patients A, B and C all treated with Primidone (NFN) received 400 mg disulfiram (Antabuse (D)) daily during the period marked by the vertical lines.

Primidone is partly converted to phenobarbitone in the organism (BUTLER 1956) and Fig. 2 shows the variations in the serum phenobarbitone concentrations in the three patients who received treatment with this drug. Some irregularities are seen, and in the case of patient C, there is a rise which rapidly evened out after withdrawal of the disulfiram.

Discussion

No large dose was given, as is often the case when disulfiram is started. If it had, the rise in the serum phenytoin concentrations would then have undoubtedly been more dramatic. As the rise continues steadily during treatment, there is no doubt that all four subjects would have been poisoned if disulfiram had been continued.

Phenytoin is converted to a large extent in the liver to 5-(p-hydroxy-phenyl)-5 phenyl hydantoin (BUTLER 1957 MAYNERT 1960), and only about 1 / of a given dose is excreted unchanged by the kidneys. It is thus reasonable to assume that parahydroxylation is inhibited by disulfiram. This could be investigated by determining the 24-hour excretion of the metabolite before and during the experiment.

The question of the effect of disulfiram on the serum concentrations of phenobarbitone has not been solved in the present investigation, as a combined effect may be involved lie on the one hand, an inhibition of the breakdown of phenobarbitone, and on the other a reduced conversion (oxygenation) of primidone to phenobarbitone. As the fluctuations observed in the concentrations of phenobarbitone only amount to about 10 /, they have hardly any therapeutic significance. Cyanamide compounds can be used as well as disulfiram in the treatment of chronic alcoholism. Whether these compounds have a corresponding effect on the breakdown of phenytoin is now being investigated

Summary

The administration of disulfiram (= antabuse) in therapeutic doses (400 mg daily) to 4 adult males who had received treatment with phenytoin over a period of years, resulted in a 100-500 / rise in the concentrations of serum phenytoin (determined by two different methods) in the course of 9 days, and no sign of levelling off of the rise in serum concentration was observed.

In view of the cases of poisoning previously observed at this hospital, phenytoin-disulfiram treatment must be regarded as involving a serious risk of phenytoin poisoning.

It must be assumed that 3 weeks will elapse after withdrawal of Antabuse, before the serum concentrations become normal.

There was only a slight effect on the phenobarbitone concentrations in the serum of 3 of the experimental subjects who were simultaneously being treated with primidone The reason for this, however may be that the oxidation of primidone to phenobarbitone is also inhibited.

Acknowledgement

I would like to thank the laboratory assistants, as well as the four patients, for their co-operation.

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From the Metabolic Division, Department of Pharmacology
University of Gothenburg, Gothenburg, Sweden

Effect of Pronethalol on the Vasodilatory and Lactic Acid Stimulating Effects of Adrenaline in the Human Forearm

By

L. Lundholm and N. Svedmyr

(Received February 10, 1966)

Pronethalol (2 isopropylamino-1 2 naphtyl ethanol = nethalide = ALDERLIN ® ICI) is an adrenergic β-receptor blocking agent that has been found to inhibit the heart-stimulating, vasodilatory and smooth-muscle relaxant effects of the catecholamines in man and other animals (BLACK & STEPHENSON 1962 DORNHORST & ROBINSON 1962). The fatty acid liberating effect of adrenaline was also inhibited by pronethalol in experiments on man, but not the hyperglycaemic effect (PILKINGTON *et al.* 1962). As a close relationship has been demonstrated between the vasodilatory and the lactic acid stimulating effects of adrenaline (LUNDHOLM 1956 LUNDHOLM & SVEDMYR 1965 LUNDHOLM, MOHME-LUNDHOLM & SVEDMYR 1966), the inability of pronethalol to block the hyperglycaemic effect of adrenaline was somewhat surprising It has been shown, however that pronethalol inhibits the phosphorylase-activating effects of adrenaline and isoprenaline in isolated rat diaphragm (MOHME-LUNDHOLM & SVEDMYR 1964) It was therefore considered of interest to attempt to determine whether or not pronethalol affected the lactic acid stimulating action of adrenaline in man at a concentration that blocked its vasodilatory effect.

Methods

The experiments were performed on 5 voluntary male subjects aged 20–25 years. The subjects had fasted since the previous evening. They lay on a bed in thermostatically controlled room at 25 A fine teflon catheter was introduced under local anaesthesia into the brachial artery to be used both for continuous blood pressure recordings with Statham pressure transducer on Grass polygraph writer and for the withdrawal of blood samples. A cannula was introduced into superficial vein on the same forearm for the infusion of pronethalol and adrenaline. A further cannula was introduced into the right

cubital vein, in a distal direction, and through this a fine teflon catheter was inserted for about 3 cm in a distal direction into the *vein that, arising from the forearm muscles, connects* with the cubital vein. From this catheter blood samples were taken for determining the lactic acid content and pCO_2 of the venous blood. The right forearm was then placed in a plethysmograph, and the blood flow was determined by *venous occlusion plethysmography* as described by BARCROFT & SWAN (1953). The plethysmograph was thermostatically controlled at 35° The volume changes of the plethysmograph were recorded by a Grass volume transducer PT 5-A on a Grass polygraph. The heart rate was recorded by a Grass cardiotachygraph. The subject was given 1 ml 5% heparin intravenously before beginning the experiment.

The blood flow in the forearm was recorded every minute during a basal 30 minute period, and basal samples of arterial and venous blood were taken during this period. Adrenaline was then infused i.v for 30 minutes, by means of an infusion apparatus, at a dose of 0.1 μg/kg/min. (= first infusion). The adrenaline solution was diluted with 0.9% NaCl to which was added 0.1% ascorbic acid for stabilization of the adrenaline. After 3, 10 and 30 min, adrenaline infusion samples of arterial and venous blood were taken simultaneously and arterial blood were also taken after 20, 40, 50, 60, 70, 80 and 90 min. After 90 min. an infusion of pronethalol (ALDERLIN ® ICI) at a dose of 1 mg/kg was given i.v for 10 min. Samples of arterial and venous blood were again taken, and a further adrenaline infusion was given for 30 min. (= second infusion). Blood samples were then taken as during the previous infusion.

The lactic acid contents of the blood were determined enzymatically by the method of LUNDHOLM, MOHME LUNDHOLM & VAMOS (1963). The plasma pCO_2 was determined by ASTRUP's method (1956).

The lactic acid elimination from the forearm was estimated from the product of the difference in lactic acid concentration between venous and arterial blood, and the mean of the blood flow values obtained 1 minute before and 1 minute after withdrawal of the blood samples for the lactic acid determination.

Results

The mean effects on the *blood flow* in the forearm induced by adrenaline and by adrenaline along with pronethalol may be seen from fig. 1A and table 1 During the first infusion of adrenaline the blood flow increased progressively to more than twice the basal value, and the raised value was maintained for about 30 min after the end of the infusion The pronethalol infusion possibly lowered the blood flow somewhat (fig. 1B), and the decrease tended to become rather more pronounced during the first few minutes of the adrenaline infusion The blood flow then tended to rise after about 15 minutes infusion but the increase was considerably smaller than before the pronethalol. Since the mean blood pressure rose somewhat at the same time (fig. 2) the blood flow increase after pronethalol with adrenaline was probably partly passive

During the infusion the *heart rate* rose from 65 to about 80/min. (fig. 2). During the pronethalol infusion the pulse dropped and the decrease became still more pronounced during the second adrenaline infusion (table 1)

Table 1

Effect f pronethalol (1 mg/kg) and adrenaline (0.1 µg/kg/min.) and adrenaline + pronethalol on circulatory variables and forearm metabolism of man. Adrenaline effect = mean change from the basal values of 3 determinations after 3, 10 and 30 min. infusion of adrenaline. Mean of 5 tests ± S.E.M.

	Basal values before pronethalol	Basal values 10 min. after pronethalol	Difference-pronethalol effect	Adrenaline effect at 3, 10 and 30 min. Change from basal values		
				Mean effect before pronethalol	Mean effect after pronethalol	Difference
Blood flow ml/100 g tissue/min.	2.0 ± 0.15	2.5 ± 0.42	0.37 ± 0.25	3.32 ± 0.51 P < 0.001	0.44 ± 0.22	2.8 ± 0.55 P < 0.01
Heart rate/min.	86.8 ± 0.97	62.0 ± 1 1	−4.8 ± 1 7 P < 0.05	+17 7 ± 1 9 P < 0.001	−4.3 ± 1.2 P < 0.01	22.0 ± 2.2 P < 0.001
Syst. blood pressure mm Hg	143 ± 4.3	138 ± 5.3	−5.0 ± 3.6	12.1 ± 2.3 P < 0.001	14.6 ± 2.1 P < 0.001	2.5 ± 3 1
Diast. blood pressure mm Hg	81 ± 4.6	80 ± 6.0	−1.6 ± 1.5	−7.3 ± 1 6 P < 0.001	12.9 ± 1.5 P < 0.001	20.2 ± 2.2 P < 0.001
Lactic acid elimination mg/100 g/min.	0.014 ± 0.011			0.123 ± 0.037 P < 0.01	0.021 ± 0.013	0.102 ± 0.038 P < 0 02
Lactic acid content of arterial blood, µg/ml	66 ± 3	–		58 ± 18 P < 0.01	5 ± 4	52 ± 16 P < 0.01
of arterial plasma mm Hg	41.0 ± 0.8	–	–	1 7 ± 0.6 P < 0.02	−0.7 ± 0.4	2.9 ± 0.8 P < 0.02
venous plasma mm Hg	41 7 ± 0.7	–	–	2.8 ± 1.2 P < 0.05	−0.2 ± 0.4	2.6 ± 1 1 P < 0.05

cubital vein, in a distal direction, and through this a fine teflon catheter was inserted for about 3 cm in a distal direction into the vein that, arising from the forearm muscles, connects with the cubital vein. From this catheter blood samples were taken for determining the lactic acid content and pCO_2 of the venous blood. The right forearm was then placed in a plethysmograph, and the blood flow was determined by venous occlusion plethysmography as described by BARCROFT & SWAN (1953) The plethysmograph was thermostatically controlled at 35 The volume changes of the plethysmograph were recorded by a Grass volume transducer PT 5-A on a Grass polygraph. The heart rate was recorded by a Grass cardiotachygraph. Th subject was given 1 ml 5 % heparin intravenously before beginning the experiment.

The blood flow in the forearm was recorded every minute during basal 30 minute period, and basal samples of arterial and venous blood were taken during this period. Adrenaline was then infused i.v. for 30 minutes, by means of an infusion apparatus, at dose of 0.1 μg/kg/min. (= first infusion). The adrenaline solution was diluted with 0.9 % NaCl to which was added 0.1 % ascorbic cid for stabilization of the adrenaline. After 3, 10 and 30 min, adrenaline infusion samples of arterial and venous blood were taken simultaneously and arterial blood wer also taken after 20, 40, 50, 60, 70, 80 and 90 min. After 90 min. an infusion of pronethalol (ALDERLIN ® ICI) at a dose of 1 mg/kg was given i.v. for 10 min. Samples of arterial and venous blood were again taken, and further adrenaline infusion was given for 30 min. (= second infusion). Blood samples were then taken as during th previous infusion.

The lactic acid contents of the blood were determined enzymatically by the method of LUNDHOLM, MOHME LUNDHOLM & VAMOS (1963). The plasma pCO_2 was determined by ASTRUP's method (1956).

The lactic acid elimination from the forearm was estimated from the product of the difference in lactic acid concentration between venous and arterial blood, and the mean of the blood flow values obtained 1 minute before and 1 minute after withdrawal of the blood samples for the lactic acid determination.

Results

The mean effects on the *blood flow* in the forearm induced by adrenaline and by adrenaline along with pronethalol may be seen from fig. 1A and table 1 During the first infusion of adrenaline the blood flow increased progressively to more than twice the basal value, and the raised value was maintained for about 30 min after the end of the infusion. The pronethalol infusion possibly lowered the blood flow somewhat (fig. 1B), and the decrease tended to become rather more pronounced during the first few minutes of the adrenaline infusion. The blood flow then tended to rise after about 15 minutes infusion, but the increase was considerably smaller than before the pronethalol Since the mean blood pressure rose somewhat at the same time (fig. 2) the blood flow increase after pronethalol with adrenaline was probably partly passive.

During the infusion the *heart rate* rose from 65 to about 80/mm. (fig. 2). During the pronethalol infusion the pulse dropped and the decrease became still more pronounced during the second adrenaline infusion (table 1).

During the first minute of the adrenaline infusion both systolic and diastolic *blood pressures* decreased. The systolic blood pressure then rose, while *the diastolic remained* at a lower level. The mean blood pressure changed little except during the first minute. Pronethalol tended to decrease both systolic and diastolic blood pressures. Adrenaline administered after pronethalol increased both, and also the mean blood pressure, but without exerting an initial depressant effect (fig. 2).

Lactic acid elimination from the forearm increased progressively during the adrenaline infusion (fig. 3); after 30 minutes infusion it was more *than 10 times the original value. Pronethalol inhibited the effect of* adrenaline on lactic acid elimination during the first 10 minutes. After 30 minutes there was a trend towards increased elimination, but not a statistically significant difference. The inhibition of lactic acid elimination by pronethalol was statistically significant (table 1).

Adrenaline increased the *lactic acid content of the arterial blood* from an average of 60 to an average of 140 µg/ml after 30 minutes infusion, and this content reached its basal value 60 minutes after the end of the infusion. Pronethalol considerably inhibited the increase in lactic acid content of the arterial blood after adrenaline (fig. 4). The extent of this action was statistically significant (table 1).

The CO_2 tension in the arterial and venous blood was also studied. During the first adrenaline infusion the pCO_2 in the arterial blood rose,

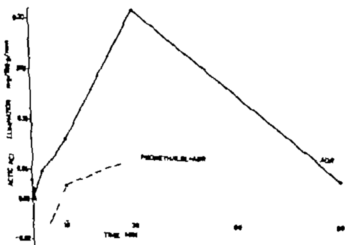


Fig. 3. Effect of adrenaline on lactic acid elimination from the forearm muscle. Effect of pronethalol on the adrenaline action. The elimination of lactic acid has been calculated from the product of the venous-arterial lactic acid difference and the mean of the blood flow values 1 min. before and 1 min. after withdrawal of the blood samples. Each point is the mean of 5 tests.

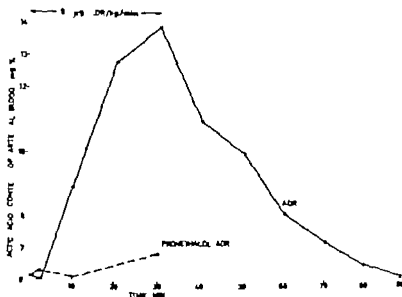


Fig. 4. The effect of adrenaline and pronethalol + adrenaline on the lactic acid content of the arterial blood. Mean of 5 tests.

the increase being most marked after 10 minutes infusion (fig. 5). The pCO_2 also increased in the venous blood from the muscle during the adrenaline infusion. No clear increase in pCO_2 was observed during the infusion of adrenaline after pretreatment with pronethalol either in the arterial or the venous blood.

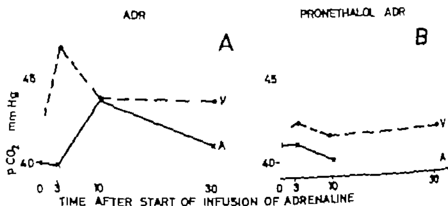


Fig. 5. A. The effect of adrenaline on the pCO_2 of the arterial blood (A) and the venous blood from the forearm muscle.
B. The effect of adrenaline when preceded by pronethalol administration. Mean of 5 test.

Discussion

LUNDHOLM & SVEDMYR (1966) found in experiments on man that the dilatory effect of adrenaline on the forearm vessels was associated with stimulation of lactic acid production, oxygen consumption and CO_2 production in the forearm, and also with an increase in the pCO_2 in arterial and venous blood from the forearm muscles. It was believed that this stimulation of metabolic processes in skeletal muscles was of importance for the vasodilatory effect of adrenaline. It was assumed that the vasodilatation was induced by the increased pCO_2 in the blood and tissues.

In the light of the close relationship between the vasodilatory and metabolic effects of adrenaline, indicated by these previous experiments, it seems probable that the blocking action by pronethalol on the lactic-acid stimulating of adrenaline was of significance for the inhibition of the vasodilatory effect of adrenaline. The elevating effect of adrenaline on the pCO_2 in arterial and venous blood was probably induced partly by stimulation effect of oxygen consumption and partly as a result of an increased lactic acid production in the tissues (LUNDHOLM & SVEDMYR 1966) In these experiments pronethalol blocked the stimulatory effect of adrenaline on lactic acid production in the forearm and also the general increase in lactic acid content of the arterial blood. Pronethalol probably also inhibited the stimulation of oxygen consumption by adrenaline in the forearm, an assumption supported by some preliminary experiments (LUNDHOLM & SVEDMYR, unpublished results) Pronethalol was also found to inhibit the stimulation of oxygen consumption, lactic acid production and FFA-mobilization by adrenaline in experiments on rabbits (SVEDMYR, unpublished results).

Summary

Pronethalol (1 mg/kg, i.v.), an adrenergic β-receptor blocking agent, almost completely inhibited the dilatory effect of adrenaline (0.1 μg/kg/min.) on the vessels of the forearm Pronethalol also inhibited the stimulating effect of adrenaline on lactic acid elimination in the muscle as well as the increase in the lactic acid content of arterial blood after adrenaline The elevating effect of adrenaline on the pCO_2 in the arterial and venous blood arising from the muscle was also blocked by pronethalol. It is suggested that the blockade of the vasodilatory effect of adrenaline is dependent on the inhibition of its metabolic effects by pronethalol. The stimulating effect of adrenaline on the heart rate was inhibited by pronethalol and changed to a reduction. Before pronethalol adrenaline

the systolic blood pressure and reduced the diastolic, so that the mean blood pressure was hardly changed. After pronethalol adrenaline raised both, so that the mean blood pressure increased.

Acknowledgement

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From The Department of Pharmacology Royal Danish School of Pharmacy (Professor Erik Jacobsen M.D.) and from The Department of Pharmacology University of Copenhagen (Professor Knud O. Møller M.D.), Copenhagen, Denmark

Intestinal Absorption of Quinine from Enteric Coated Tablets

By

Søren Rasmussen

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The fact that many drugs cause gastric irritation when administrated orally has a.o. increased the interest in enteric coated preparations i.e. granulates, pills or tablets coated with a layer that allows passage through the stomach and disintegration in the upper part of the intestine (DRAGSTED 1958 MORRISON & CAMPBELL 1965

Numerous papers have been published on the properties of such preparations, especially on methods for examining the *in vitro* disintegration. Most pharmacopoieas include tests for their enteric coated preparations, all based on the principle that they remain intact when placed in an acid solution and disintegrate when placed in a neutral or slightly alkaline one. These pharmacopoiee methods have been rewiewed (in danish) by NUPPENAU (1965)

Reports of *in vivo* studies are less frequent. Two different types of techniques have been used Some have measured the *in vivo* disintegration time of $BaSO_4$-containing enteric coated tablets by X-rays. The experiments of this type have been rewiewed by HODGE *et al.* (1944) and LAZARUS & COOPER (1959) The method there has given proportionality between the *in vitro* and the *in vivo* disintegration times (WAGNER *et al* 1958 and WAGNER *et al* 1960)

A different method of examining the time of disintegration involves measuring when the active substance from the tablets appears in the blood It has the advantage that it not only gives a measure of the disintegration time but also makes it possible to determine the amount absorbed

The absorption of acetylsalicylic acid from enteric coated preparations has been investigated by LEONARDS & LEVY (1965), HOLLISTER & KANTER (1965) and by CLARK & LASAGNA (1965). The last named authors found less complete absorption from the coated tablets than from the uncoated tablets. Quinidine absorption has been investigated by SAMPSON *et al.* (1952), who in some experiments found a failure in disintegration and a decreased therapeutic effect from the enteric coated preparations.

A corresponding failure in absorption was reported by HAAPANEN & RIKKOLA (1964) who worked with p-aminosalicylic acid. In a note on oral potassium salts, PIRNIE & STAFFURTH (1961) report a complete failure of absorption of potassium from an enteric coated preparation, though the preparation fulfilled the *in vitro* tests of the British Pharmacopoeia.

WYNN & LONDON (1963) examined the absorption from enteric coated tablets containing NaCl and KCl and found complete absorption from all.

In these experiments the absorption was measured from commercial preparations, and no information on the nature of the coating material and the thickness of the coating layer are given in the reports. From the investigations it appears that *in vitro* examinations may give some information on the preparations, but that they cannot predict how the absorption *in vivo* of the active substance will proceed.

Moreover no systematic examination of the correlation between the delay in disintegration time and the amount absorbed has been found in the literature.

In our study the effect of the thickness of the coating layer on the absorption of different drugs from tablets coated with cellulose acetate phthalate has been examined. This paper reports the results of experiments on the absorption of quinine hydrochloride, a substance well suited for the investigation, because it is easily determined in the plasma at non-toxic concentrations.

Methods

Eighteen healthy adult volunteers of both sexes served as experimental subjects. Their diet was not restricted, and during the experiments they carried out their usual work in the laboratory. Only males took part in the experiments in which urinary excretion was measured.

Blood samples were drawn from the ear-lobe immediately before the test dose and later at intervals of approximately one hour. In some experiments the urine was collected for 10–12 hours before the administration and at intervals of 1–6 hours up to 48 hours after.

Tablets had the compositions given below

30 mg tablets. N 100,000. Diameter 6 mm.

Quinine hydrochloride	5,000 g
Starch	4,000 g
Spiritus gelatinosae 5% Ph. Nord. (1965a)	sufficient quantity
Talc	ad 10,000 g

500 mg tablets. No 10,000. Diameter 12 mm.

Quinine hydrochloride	5 000 g
Starch	1,500 g
Spiritus gelatinae 5 % Ph Nord. (1965)	a sufficient quantity
Talc	ad 7 000 g

From these two batches, subbatches of 4000 and 2000, respectively were coated. The coating was made with *vernix enterosolubile* Ph.Nord. (1965 b), consisting of

Cellulose acetate phthalate	200 g
Oleum ricini	10 g
Acetone	1 790 g

T compare the thicknesses of the coating on the tablets of the different sizes, it was calculated as mg vernix per mm² surface. The average difference between the weight of coated and uncoated tablets was determined on 200 tablets, and the surface f each tablet was calculated geometrically from its dimensions.

Preparations were made with various thickness of the coating layer as seen from table 1. Quinine was determined in plasma by the method of Brodie & Udenfriend (1943), quantitatively modified to allow determinatio in 0.1 ml plasma. The readings were made a Photovolt fluorometer with the filters P = Hg 1 and S = B 540 and compared with standard curves for known amounts of quinine dissolved in human plasma, in order to compensate for the quenching effect of the chloride ions.

Table 1

Thickness of the coating layer the different preparations expressed as mg vernix per mm² surface

Preparation o	mg/mm²
50 mg tablets	
5	0.0423
6	0.0315
7	0.130
8	0.0324
9	0 0687
15	0.178
500 mg tablets	
11	0.0336
12	0.0585
13	0.121
14	0.242

Preparation no 2 and 10 are uncoated tablets of 50 nd 500 mg, respectively from which all the coated preparations were manufactured.

In the urine analysis, the extraction procedure of Brodie *et al* (1947), which was first tried, showed no advantage over a determination directly on the urine identical to that for plasma. Standards were dissolved in the urine collected before the administration, which eliminate most of the basic fluorescence in the urine. The blank fluorescence in the samples could vary in amounts corresponding to a excretion of 0–9 mg quinine in 48 hours. Haag *et al* (1943) report an excretion" of 0–4 mg in 24 hours. The effect of the variations in this blank value was estimated as negligable compared with the amount excreted after the doses given.

Results

Plasma concentrations after administration of 0.2, 0.3 and 0.5 g quinine HCl in aqueous solutions to the same person are shown in fig. 1 The concentrations of plasma quinine determined 2 hours after the same dose varied somewhat from person to person, after 0.5 g, between 2.36 and 5 30 µg/ml with an average of 4 12 µg/ml ± 20 6 / (s) d.f = 8. The variation from time to time in the same person was considerably smaller s = 10 7 / d.f = 24 The graphically calculated rate of elimination showed variations of ±13 6 / (s) d.f = 16, within individuals and ±36 / (s) d.f = 24 between individuals.

The results from all experiments with various doses are given in table 2 A clear proportionality is seen between the dose administrated and the plasma concentration measured at any time in the test period. This means that it is possible to estimate the amount absorbed from a given dose when equilibrium of the distribution in the body is reached, if the plasma concentration is compared with that obtained at the same time after a known dose in aqueous solution

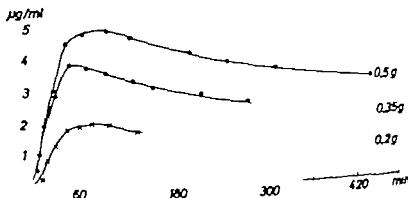


Fig. 1 Plasma concentrations of quinine HCl after oral intake of 0.50, 0 35 and 0.20 g quinine HCl in aqueous solution. (Experimental subject no 1).

Abscissa Minutes after the intake.
Ordinate Concentration i µg/ml.

Table 2

Plasma concentrations at different times after the administration of 0.20 and 0.30 g quinine hydrochlorid in aqueous solution, as percentages of the corresponding concentration after administration of 0.5 g (= 100 % ± 10.7 %). Figures in brackets are the ranges.

Hours after ingestion	Plasma concentration: Dose 200 mg (= 5)	300 mg (= 3)
2	38 % (37–40)	72 % (71 73)
3	38 % (37–41)	72 % (70–76)
4	39 % (35–42)	72 % (68–79)
5	39 % (28–42)	68 % (66–71)
6	36 % (27–41)	70 % (67–76)
7	36 % (29–40)	69 % (66–74)
Average	38.0 % ± 4.1 % (s)	70.5 % ± 3.9 % (s)
Dose as percentage of 500 mg	40 %	70 %

Fig. 2 shows an example of plasma concentrations measured in experiments in which 0.5 g quinine HCl was given as one single dose (1) or as 10 doses of 50 mg each at intervals of 10 minutes (2) or of 30 minutes (3). The results from all experiments of this type are collected in table 3 The plasma concentration measured when divided doses are given naturally increases more slowly than after one single full dose, and the maximum is reached about one hour after the last fraction is given, but at this point the concentration reaches or often even exceeds that from the single dose.

Table 3

Plasma concentrations after intak f 0.5 g quinine hydrochloride as 10 × 50 mg in aqueous solution at intervals of 10, 20 and 30 minutes between divided doses as percentages of the corresponding concentration after intake of 0.5 g as single dose in queous solutions (= 100 % ± 10.7 % s)

Hours after the first dose	Plasma concentrations (see text) Min. between divided doses 10 (= 2)	20 (n = 1)	30 (n = 2)
2	76–81 %	60 %	36–49 %
3	90–110	84 %	57 73 %
4	91 117 %	102 %	78–102 %
5	89–110 %	93 %	100–134 %
6	87–108 %	87 %	108–132 %
7	87–100 %	86 %	100–118 %
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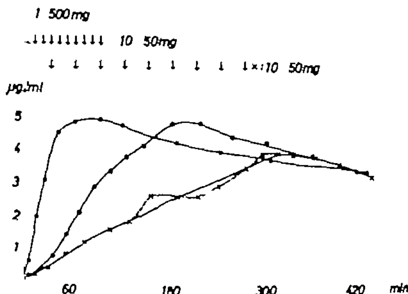


Fig. 2. Plasma concentrations after intake of 0.5 g quinine HCl in aqueous solution. (Experimental subject no 1).

● = 500 mg at zero time.
○ = 10 × 50 mg at 10 minute intervals.
× = 10 × 50 mg at 30 minute intervals.

The arrows indicate the time of intake.
Abscissa Time in minutes.
Ordinate Concentration in µg/ml

It is further seen that slower the absorption the less steep is the slope of the ascending branch of the plasma concentration curve.

In fig. 3 these slopes, recorded from all experiments similar to that represented in fig. 2, are plotted against the time between the doses, which is inversely proportional to the rate of absorption. As seen, there is proportionality between the slope and the rate of absorption at least when it is completed within 5–6 hours.

This means that it is possible to estimate the least expected peak concentration of a given dose when the rate of absorption and the curve from ingestion of a standard dose of 0.5 g quinine HCl to the same person are known. Similarly it is possible to estimate the amount absorbed from a given dose, if the rate of absorption and the elimination curve of a standard dose are known.

As will be seen below the absorption from the coated tablets may be prolonged over a considerable time. The principle just described is applied here in order to estimate approximately how much of a given dose

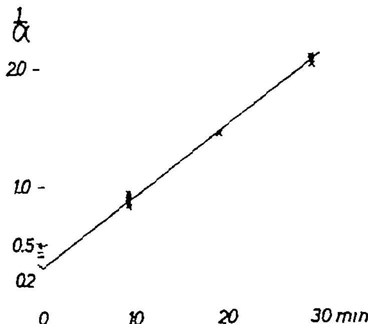


Fig 3. Relation between the reciproced, recorded slope (α) of the ascending branch of the plasma concentration curve and the rate of absorption, measured as the intervals between the divided doses.

Abscissa Intervals between the dose fractions in minutes.
Ordinate 1/α.

has in fact been absorbed. Examples of the procedure are given in fig. 4A to D From the test itself (4A and C) the maximal plasma concentration a, is recorded. The slope of the line drawn through the ascending branch of the concentration curve is conveniently determined graphically A line is drawn parallel to this through the zero point on the curve obtained from administering a standard dose of 0.5 g quinine HCl in aqueous solution to the same person. The point where this line intersects the curve, represents the concentration b which should have been reached provided that the total dose has been absorbed The approximate amount absorbed is calculated by the formula $Rc = \frac{a}{b}$ This ratio represents the part of the dose of 0.5 g that is absorbed. If complete absorption takes place, the value should be equal to or exceed one.

Rc-values calculated from tests with known amounts in aqueous solutions are given in fig. 5 The Rc values calculated in this way give measures of the amounts absorbed relative to the absorption from 0.5 g in aqueous solution sufficiently reliable to be used in our investigation.

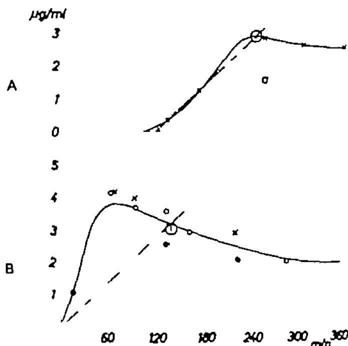


Fig. 4, A to D Plasma concentrations after intake of 0 5 g quinine HCl i different preparations.

4 A. Intake of 0.5 g in tablets coated with 0.0687 mg/mm² (preparation no. 9). Experimental subject no. 3

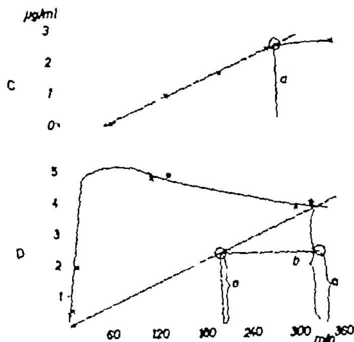
4 B. ● = 0.5 g i aqueous solution.
○ = 0.5 g i queous solution.
× = 0.5 g in uncoated tablets (preparation no. 2). Experimental subject no. 3.

Abscissa Time in minutes after intake.
Ordinate Concentration in µg/ml.

The broken lines in 4 B and D represent the measured slope of the ascending branch of the curves in fig. 4 A and C, respectively displaced t the zero point. The letters a and b indicate the concentrations mentioned i th text from which the amount absorbed, the Rc-values are calculated. For further explanation see text.

From tests on ingestion of uncoated tablets, the total amount always being 0 5 g quinine HCl, the calculated Rc values were found to average 1 01 ± 0.044 (s.e.m.) n = 11 which means that the absorption *from* the uncoated tablets is the same as from the aqueous solution.

The plasma concentration curves in fig. 4 *A* and *C* obtained from two administrations of the coated preparation no 9 show a delay in the onset of absorption, which is not found in tests with uncoated tablets. The nearest whole hour after the ingestion when detectable amounts of quinine



4C. Intake of 0.5 g in tablets coated with 0.0687 mg/mm² (preparation no. 9). Experimental subject no. 2.

4D. ● = 0.5 g in aqueous solution.
× = 0.5 g in uncoated tablets (preparation no. 2). Experimental subject no. 2.

are found in the plasma is taken as the *in vivo* disintegration time of the preparation.

In fig. 6 the *in vivo* disintegration times are plotted against the thickness of the coating layer and found proportional to it. From the curves in fig. 4A and C it is further seen that the absorption rate from the coated tablets is lower than from the aqueous solution. The slopes of the ascending branches from all tests with coated and uncoated tablets are shown in fig. 7 plotted against their corresponding *in vivo* disintegration times, indicated as the interval between two plasma determinations in which the first increase in plasma quinine was found. From the figure it is seen that if the *in vivo* disintegration time exceeds one hour the slope is significantly lower than that found for the uncoated tablets ($P < 0.001$). This delay in absorption rate could be explained as a difference in the disintegration times of the tablets if all tests involved administration of 10 tablets of 50 mg each, but this is disproved by the fact that a similar decrease was found in tests with 500 mg coated tablets (broken lines in fig. 7)

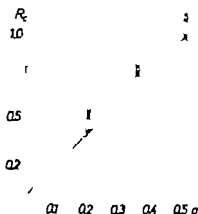


Fig. 5 Absorbed amounts, calculated as Rc-values in relation to the doses ingested.

Abscissa Dose in g.
Ordinate Rc-values. The value 1.0 indicates absorption equal to the absorption from 0.5 g in aqueous solution.

Broken line Theoretical Rc-values $Rc = \frac{\text{dose in mg}}{500}$

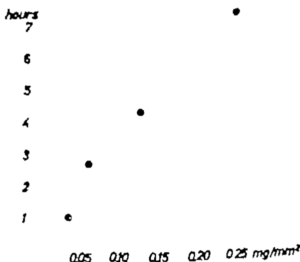


Fig. 6. *In vivo* disintegration time in relation to the thickness f the coating layer

Abscissa Thickness of the coating layer in mg verulx per mm² tablet surface.
Ordinate *In vivo* disintegration time in hours.

⊗ dose given as one 500 mg tablet
× dose given as 10 tablets of 50 mg each.

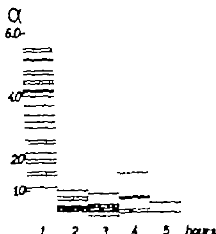


Fig. 7 The slope of the ascending branch α, of the plasma concentration curve, recorded from all tests with uncoated and coated tablets, in relation to the *in vivo* disintegration time.

Abscissa *I* vivo disintegration time in whole hours.
Ordinate Slope I arbitrary units.
Broken lines Tests with 500 mg tablets.
Unbroken lines Tests with 10 × 50 mg tablets.

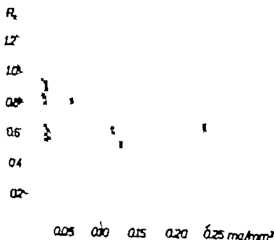


Fig. 8. Relation between the amount absorbed, calculated as Rc-values, and the thickness of the coating

Abscissa Thickness of the layer in mg/mm²
Ordinate Calculated Rc-values.

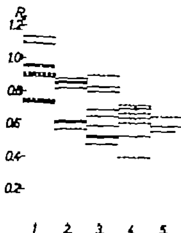


Fig. 9 Relation between the bsorbed amount, calculated as Rc values, and the *in vivo* disintegration time.

Abscissa *I vivo* disintegration time in whole hours.
Ordinat Calculated Rc values.
Broken lines: Tests with 500 mg tablets.
Unbroken lines Tests with 10 × 50 mg tablets.

The amount absorbed, calculated as the Rc values, from all tests with tablets, are plotted in fig. 8 against the thickness of the coating layer and found to decrease with increasing thickness. With small tablets only this might be explained by assuming that some of tablets did not disintegrate, but results from 500 mg coated tablets show a similar decrease.

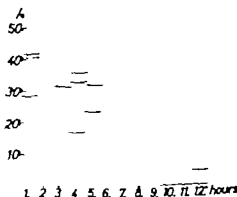


Fig. 10. Excretio in the urine as percentage of the ingested dose (500 mg) in relation t the *in* *ve* disintegration time.

Abscissa: *I vivo* disintegration time in whole hours.
Ordinate Excreted amount as percentage of the dose, 500 mg.
Dotted line: Test with 10 uncoated tablets of 50 mg each (preparation no. 2) administrated as 10 singl ingestions at 30 minut intervals.

Fig. 9 shows the relation between the delay in onset of absorption – the *in vivo* disintegration time – and the Rc values. With increasing *in vivo* disintegration time the amount absorbed is decreased.

Results of determinating the urinary excretion are shown in fig. 10, where the excretion, as a percentage of the dose administered, is plotted against the recorded *in vivo* disintegration time. With an increase in the latter the amount excreted is seen to decrease. This decrease cannot be due to the decrease in the absorption rate found with the increasing *in vivo* disintegration time, as one of the highest excretions was found in a test in which the dose was administrated as 10 uncoated tablets of 50 mg each ingested one by one at intervals of 30 minutes (the dotted line in fig. 10)

Discussion

From the urinary excretion (fig. 10) and the calculated Rc values in fig. 9 the Rc-value seems to be a reliable measure of the amount absorbed

As the rate of absorption and the amount absorbed were found to be the same from aqueous solutions and the uncoated tablets, the observed differences in the absorption pattern for the coated tablets must be caused by the coating layer With increasing thickness of the vernix layer an increased *in vivo* disintegration time is found. Stomach retention, such as that found in patients kept in a supine position during the experiments, as reported by PRAETORIUS & FABER (1950), is not a likely explanation of the delayed disintegration observed here, as the experimental subjects were normally active during the experiments. Moreover stomach retention would not explain the decrease in rate of absorption and the decrease in amount absorbed found in the experiments reported here.

The slow absorption rate and the decrease in amount absorbed with increasing thickness of the coating is observed both with the dose given as 10 tablets of 50 mg each and with the dose given as a single coated tablet of 500 mg and can thus not be due to a difference in the disintegration times of the 50 mg tablets.

Apparently the coated tablets disintegrate in the lower parts of the small intestine where quinine is more slowly and less completely absorbed.

A difference in absorption rate from the various parts of the small intestine is well known for substances actively absorbed, but is not an established fact for substances that, like quinine, must be assumed to be absorbed by simple diffusion processes. However a few indications that this might be so are found in litterature.

SJÖGREN & ÖSTHOLM (1961) investigated the absorption of different drugs from a sustained release preparation and found creatinine and potassium penicillin-V less completely absorbed than from conventional

tablets, although both substances were completely released *in vitro*. These authors suggests a decreased power of absorption ability by the lower parts of the intestine as a possible explanation.

Creatinine absorption from a different prolonged action preparation (Gitter" tablets) has been investigated by LUNDHOLM & SVEDMYR (1963), who found that the dose had to be doubled in order to obtain the same plasma concentration and urinary excretion as after a dose of conventional tablets. Neither SJÖGREN & ÖSTHOLM nor LUNDHOLM & SVEDMYR have compared the absorption rate with that after divided dosage.

The experiments reported here were conducted with quinine hydrochloride and the results must not be generalized. None the less, they indicate that enteric coated or sustained release preparations cannot be used therapeutically before the effect of the delayed release on absorption is known for each particular drug. Evaluation of such preparations cannot be based on *in vitro* tests alone

Summary

The effect of the thickness of the coating on the absorption of quinine hydrochloride from tablets coated with cellulose acetate phthalate has been investigated. A method for calculating the amount absorbed from the plasma concentration curve is described, and the calculated values are compared with the urinary excretion. With the coated tablets the *in vivo* disintegration time was found to increase in proportion to the thickness of the coating. The rate of absorption was decreased with increasing layer thickness and generally also the amount absorbed The findings suggest that the ability to absorb quinine is decreased in the distal parts of the intestine.

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From The First Medical Service, Sahlgrenska sjukhuset, University of Göteborg and Hässle Research Laboratories, Göteborg, Sweden

Effect of Isopropylnormetadrenaline and a Catechol-O-Methyltransferase Inhibitor on Adipose Tissue Metabolism In Vitro

By

Per Björntorp and Hans Corrodi

(Received January 14, 1966)

Adrenaline and noradrenaline increase glucose uptake by adipose tissue *in vitro* It has been suggested that this increase is secondary to the increase in fatty acid content of the system, caused by increase in lipolysis. Addition of fatty acids to the system causes the same effect on glucose metabolism of adipose tissue as do the catecholamines mentioned (LEBOEF *et al* 1959 LYNN *et al* 1960 CAHILL *et al* 1960 LEBOEF & CAHILL 1961).

Recently however evidence has been presented to suggest that the increase in glucose uptake by adipose tissue, dependent on catecholamines and related substances, is not solely a secondary effect to the increase in fatty acid concentration Thus LOVE *et al* (1963) found an increase in glucose uptake after the addition of DL 1-(2 4-dichlorophenyl)-2-t butylaminoethanol (DCB) though the fatty acid content of the system was not increased. Further it has earlier been shown that a β-adrenergic blocking agent (nethalide) seems to inhibit the noradrenaline increase of glucose uptake at concentrations at which the increase of lipolysis is not inhibited. The separation of these two effects of noradrenaline seems to suggest that the catecholamine effect on glucose metabolism is not only secondary to changes in lipolysis (BJÖRNTORP 1964)

In the work reported here, the effect of isopropylnormetadrenaline (DL 1-(3 methoxy-4 -hydroxyphenyl)-2-isopropylaminoethanol) on adipose tissue metabolism has been studied and shown to increase lipolysis and fatty acid concentration little or not at all, whereas glucose uptake is increased. Further a catechol-O-methyl transferase inhibitor (H 22/54

α-n-propyl-3,4-dihydroxyphenylacetamide) (CARLSSON *et al.* 1963) inhibited glucose uptake increase caused by isoproterenol without affecting lipolysis or the fatty acid content of the tissue significantly.

Experimental Procedure

200–300 g Wistar rats were fasted overnight, killed by a blow on the head and beheaded, and the epididymal fat pads were removed. Distal ends were used, divided each into three approximately equal parts, rapidly weighed and incubated.

Incubations for measurements of fatty acid and glycerol release were performed in siliconized cylindrical glass tubes in 5 ml Krebs-Ringer bicarbonate buffer with 4% albumin (Armour Fraction V Bovine Serum Albumin), pH 7.4, temperature 37.0°. Glucose was added as indicated in the text. Measured portions for fatty acid (DOLE 1956) and glycerol (LAMBERT & NEISH 1950) determinations were removed after 10 minutes of temperature equilibration and at times stated for each experiment.

Glucose uptake was measured in 1 ml of the medium mentioned with 11 mM glucose, now incubated in cylindrical 5 cm high polyethylene tubes with a bottom diameter of 1 cm. These tubes were sealed with polyethylene cap, and incubated lying down in the direction of the movement back and forth in the incubation bath to insure complete mixing. Glucose concentration in the medium was determined by an enzymatic method (LEVIN & LINDE 1962).

Noradrenaline (Astra) and isoproterenol were used as the bitartrates, isopropylnormetadrenaline and normetadrenaline as their hydrochlorides.

Results

Table I gives the results of incubation with noradrenaline at 5×10^{-7} M concentration and isopropylnormetadrenaline at this and higher concentrations, when fatty acids and glycerol release were measured after 1 hour and glucose uptake after 2 hours. Noradrenaline increased fatty acid release, glycerol release and glucose uptake significantly. Isopropyl normetadrenaline did not increase fatty acid or glycerol release at concentrations from 5×10^{-7} to 5×10^{-4} M, but glucose uptake was increased to some extent at these concentrations, significantly at 5×10^{-4} M concentration, with a trend to significant increase at 5×10^{-5} M.

Table 2 shows that isopropylnormetadrenaline did not affect fatty acid or glycerol release increased by noradrenalin at any of the concentrations tested.

Figure 1 gives the glycerol releases and glucose uptakes after different times of incubation in control tissues and in the presence of 5×10^{-7} M noradrenaline or 5×10^{-4} M isopropylnormetadrenaline. It can be seen that glycerol release was increased significantly in the noradrenaline system when compared with that in the flask without addition after 1 hour ($p < 0.05$). After 2 hours there was a trend to difference ($0.10 > P > 0.05$)

Table 1

The Effect of noradrenaline and isopropylnormetadrenaline on fatty acid and glycerol release and glucose uptake in adipose tissue *in vitro*. Means ± S.E.M

	Noradrenaline 0	Noradrenaline 5×10^{-7}M	Isopropylnormetadrenaline 5×10^{-7}M	Isopropylnormetadrenaline 5×10^{-6}M	Isopropylnormetadrenaline 5×10^{-5}M	Isopropylnormetadrenaline 5×10^{-4}M
Medium fatty acids (μEq/g/h) n = 5	3 27 ±0.76	14.50 ±1.25	3.01 ±0.51	2.73 ±0.23	2.43 ±0.57	3.56 ±0.79
Tissue fatty acids (μEq/g/h) n = 5	3 48 ±0 40	17 12 ±0.65	3 40 ±0.42	3.68 ±0 60	3.82 ±0.67	3.12 ±0.34
Glycerol release (μEq/g/h) n = 5	1 48 ±0.79	4.87 ±0.74	1.54 ±0.20	0.93 ±0.09	1.24 ±0.26	1.69 ±0.47
Glucose uptake (mg/g/h) n = 7	1 02 ±0.25	2.16 ±0.36	1.57 ±0.16	1 44 ±0.24	1 70( ) ±0.25	2 07* ±0.39

(*) 0.10 > p > 0 05 compared with 0.
p < 0 05 compared with 0.
p < 0.01 compared with 0.

Table 2

The effect of isopropylnormetadrenaline on fatty acid and glycerol release and glucose uptake stimulated by noradrenaline (5×10^{-7}M) in adipose tissue *in vitro*. Means ± S.E.M n = 5

	0	Noradrenaline plus Isopropylnormetadrenaline 0	5×10^{-7}M	5×10^{-6}M	5×10^{-5}M	5×10^{-4}M
Medium fatty acids (μEq/g/h)	5.00 ±1 46	14 64 ±2.06	16.31 ±1 71	13 84 ±1.37	17.85 ±.62	17.63 ±1 77
Tissue fatty acids (μEq/g/h)	4 23 ±0.96	16.48 ±0.49	16.90 ±0 72	17.20 ±0 81	16.40 ±0.64	16.19 ±0.49
Glycerol release (μEq/g/h)	1.89 ±1.05	6.15 ±1 67	7.29 ±0 67	74 ±1.31	6 90 ±1.00	7 11 ±1.41

– p < 0.05 compared with 0.
– p < 0.01 compared with 0.

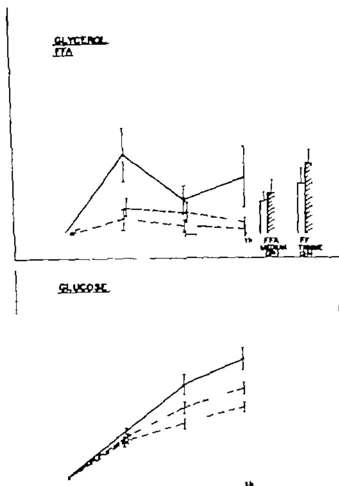


Fig. 1 Effects of Noradrenaline (5×10^{-7}M) and Isopropylnormetadrenaline (5×10^{-4}M) on glycerol release, medium and tissue fatty acid (FFA) concentrations and glucose uptake in adipose tissue *in vitro*

Continuous lines and solid columns	Noradrenaline
Broken lines and hatched columns	Isopropylnormetadrenaline.
Dotted-broken lines and open columns	No addition.

Means and SEM (vertical lines on curves) for 6–14 determinations.

Results given as µequivalents of free fatty acids or glycerol released or mg of glucose taken up per gram adipose tissue. The medium and tissue FFA concentrations were measured at 3 h.

and a significant one at 3 hours ($p < 0.05$) In the presence of Isopropylnormetadrenaline, there was a trend to significantly higher values than those of the controls at 3 hours ($0.10 > p > 0.05$), but before this time

no significant increase occurred. After 3 hours incubation, fatty acids in the medium and in the tissue had increased in the system with noradrenaline ($p < 0.05$ for medium, $p < 0.025$ for tissue) but not in the isopropylnormetadrenaline system. Glucose uptakes in the flasks with additions were not significantly increased over the control at 1 hour but after 2 hours were so increased with both noradrenaline ($p < 0.001$) and isopropylnormetadrenaline ($p < 0.05$) as well as after 3 hours ($p < 0.01$ for both).

Table 3 gives the effect of H 22/54 only. Glycerol release and medium and tissue fatty acid concentrations were increased at 5×10^{-5} M concentration.

When added to a system with isoproterenol-stimulated lipolysis and glucose uptake H 22/54 caused a significant decrease in glucose uptake at 5×10^{-4} M *concentration, but not of glycerol release and fatty acid* concentrations, as shown in table 4.

Finally normetadrenaline was tested. With this compound no effects on glycerol release, fatty acid concentration or glucose uptake in the fat pad were found, as shown in table 5.

Table 3

The effect of H22/54 on fatty acid and glycerol release and glucose uptake in adipose tissue *in vitro*. Means ± S.E.M

		H 22/54	
	0	5×10^{-5} M	5×10^{-4} M
Medium fatty acids (μEq/g/h)	3.21 ±0.49 n = 12	10.44 ±1.94 n = 6	4.11 ±0.91 n = 6
Tissue fatty acids (μEq/g/h)	3.57 ±0.40 n = 12	6.93 ±0.40 n = 6	3.97 ±0.54 n = 6
Glycerol release (μEq/g/h)	1.68 ±0.32 n = 12	3.87* ±0.37 n = 6	2.07 ±0.46 n = 6
Glucose uptake (mg/g/h)	0.96 ±0.20 n = 7	1.43 ±0.19 n = 7	1.42 ±0.16 n = 7

* p < 0,01 compared with 0.

Table 4

The effect of H22/54 on fatty acid and glycerol release and glucose uptake stimulated by isoproterenol (5×10^{-7}M) in dipose tissue *in vitro*. Means ± S.E.M.

	Isoproterenol plus H22/54		
	0	5×10^{-5}M	5×10^{-4}M
Medium fatty acids	13.68	13.10	13.10
	±1.37	±2.76	±0.52
(μEq/g/h)	n = 12	n = 6	= 12
Tissue fatty acids	17.98	16.67	16.80
	±0.79	±1.49	±0.79
(μEq/g/h)	= 12	n = 6	= 12
Glycerol release	5.60	5.36	5.35
	±0.39	±0.71	±0.39
(μEq/g/h)	= 12	n = 6	n = 12
Glucose uptake	1.75	1.57	1.09
	±0.26	±0.51	±0.14
(mg/g/h)	= 6	n = 6	= 6

= $p < 0.05$ compared with isoproterenol only

Table 5

The effect of noradrenaline and normetadrenaline on fatty acid and glycerol release and glucose uptak in adipose tissue *in vitro*. Means ± S.E.M

	Noradrenaline		Normetadrenaline			
	0	$5\times10^{-}$ M	5×10^{-7}M	5×10^{-6}M	5×10^{-5}M	5×10^{-4}M
Medium fatty acids	2.71	13.52	3.72	2.84	3.72	5.09
(μEq/g/h) = 5	±0.88	±2.09	±0.88	±0.92	±1.03	±0.99
Tissue fatty acids	3.1	16.20	3.40	2.94	3.64	3.12
(μEq/g/h) = 5	±0.42	±1.95	±0.64	±0.51	±0.70	±0.35
Glycerol release	1.21	3.97	1.32	1.03	1.19	1.82
(μEq/g/h) = 5	0.17	±0.47	±0.16	±0.27	±0.25	±0.17
Glucose uptake	1.61	2.25	1.62	1.65	1.42	1.43
(mg/g/h) = 7	±0..7	±0.51	±0.15	±0.19	±0.25	±0.24

= $p < 0.01$ compared with 0.

Discussion

It was thus demonstrated that isopropylnormetadrenaline increased glucose uptake of adipose tissue slightly whereas no significant increase in fatty acid contents of medium or tissue could be recorded, nor could any increase in glycerol output be found There was thus no evidence for a *stimulation* of the lipolytic mechanism by this compound, as evidenced by the measurements of glycerol output (VAUGHAN 1962). In spite of this, a small increase in glucose uptake was found. This, as well as the work by LOVE *et al* (1963) seems to argue against the hypothesis that the increase in glucose uptake caused by catecholamines and related substances is only secondary to their effect on the lipolytic system, causing an increase of fatty acid concentration (CAHILL *et al* 1960 LEBOEF & CAHILL 1961). It could *still* be possible, however that an increase in fatty acids occurred in a compartment of the tissue too small to allow detection by our methods, but of importance for increase in glucose uptake.

It was also demonstrated that the increase in glucose uptake caused by isoproterenol could be selectively inhibited with H 22/54 that is, could occur without effect on the increases in lipolysis and fatty acid concentration This is an effect analogous to that of a β-receptor blocking agent (nethalide), as earlier described (BJÖRNTORP 1964) Neither compound decreased basal glucose uptake on the contrary H 22/54 increased it. Thus only the catecholamine produced increase in glucose uptake is inhibited the fact that this can occur without effect on the increase in lipolysis seems to suggest that these two stimulatory properties of catecholamines may be independent.

When the lipolytic response to noradrenaline (in the form of glycerol output) and glucose uptake were measured at different times of incubation, it was found that a considerable increase in lipolytic activity occurred during the first hour but not later in this system with a small amount of noradrenaline as recorded by VAUGHAN & STEINBERG (1963) An increase in glucose uptake was found during this latter period, that is, when the fatty acid concentration of the system was increased These results confirm the earlier reported effects of increased fatty acid levels on glucose uptake in adipose tissue (CAHILL *et al* 1960 LEBOEF & CAHILL 1961)

It might be reasoned, that the 3-0-methyl derivatives of catecholamines are partly responsible for the increase in glucose uptake found in adipose tissue incubated with catecholamines, since the latter are transformed to the 3-0-methyl derivatives in several tissues (AXELROD 1959) This is, however probably of minor importance First, this does not seem to occur with noradrenaline, since its 3-0-methyl derivative was found inactive. Further the increase in glucose uptake caused by isopropyl-

normetadrenaline was much smaller than that by isoproterenol Finally small activities of catechol-0-methyltransferase have been found in adipose tissue (STOCK & WESTERMANN 1963)

Isopropylnormetadrenaline infused into the anaesthetized dog gives an increase in plasma free fatty acids and no apparent change in blood glucose (BJÖRNTORP & ÅBLAD unpublished observations) Effects corre ponding to the ones found *in vitro* can thus apparently not be observed *in vivo*

Summary

Glucose uptake in rat adipose tissue *in vitro* was slightly stimulated by isopropylnormetadrenaline without stimulation of glycerol output or increase in fatty acid concentration in the incubation medium or in the tissue. Normetadrenaline did not have a similar effect. Further the in crease in glucose uptake, caused by isoproterenol could be inhibited by H 22/54 without inhibition of the increase in glycerol output or fatty acid concentration.

Acknowledgements

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From the Section of Experimental Anaesthesiology
(Professor O Secher M D), the Department of Pharmacology
(Professor Knud O. Møller M.D.).

Protective Action of Carbon Dioxide Against Anoxia With and Without Anaesthesia

By

Bendt J Wilhjelm

(Received February 24 1966)

It has been shown previously that thiopentone (BP) (= tiomebumal NFN = thiopental WHO) increases the tolerance of mice to anoxia (ARNFRED & SECHER 1962 WILHJELM & ARNFRED 1965) Various other anaesthetics have also been shown to have a similar tolerance-increasing effect on mice (WILHJELM & ARNFRED 1965 WILHJELM 1965)

Barbituric acid derivatives depress the respiratory centre. Administration of such derivatives will therefore cause a rise in arterial pCO_2, unless artificial ventilation is established. In mice, too, administration of thiopentone (tiomebumal) will cause the pCO_2 of the arterial blood to rise (WILHJELM & LANGGÅRD 1965)

Inspiration of carbon dioxide effects an increase of cerebral blood flow in human subjects (KETY & SCHMIDT 1948) and in rhesus monkeys (REIVICH 1964) Moreover various papers have been published in which it is stated that inspiration of carbon dioxide increases the tolerance of both human subjects and animals to anoxia (KEYS, STAPP & VIOLANTE 1943 KLINE 1947 RAHN & OTIS 1947 HALL & HALL 1951).

The increased tolerance to anoxia noticed in mice after administration of thiopentone might therefore be thought due to an increased cerebral blood flow caused by a rise of arterial pCO_2 in consequence of the depressive action on the respiratory centre.

Our investigation was carried out with a view to studying the effect of inspiring carbon dioxide on the survival of mice exposed at the same time to anoxia.

Further we studied the effect of carbon dioxide on the length of survival of mice anaesthetized with thiopentone or halothane and also exposed to anoxia.

Material and Method

As experimental animals we used male albino mice ranging in weight from 25 to 30 g. The experimental procedure has been described in a previous paper (WILHJELM & ANDERSEN 1965). The experiment proceeded in two stages. The first comprised investigations into the effect of carbon dioxide on the survival of mice exposed to anoxia. While placing the mice in the bottles kept in a thermostat, we perfused the system continuously with 20% oxygen and 80% nitrogen. Thereafter the gas mixture was changed to one containing 5% oxygen and carbon dioxide (carbon dioxidum NFN) at the concentration tested in the individual experiments, with nitrogen added to 100%.

A group of 50 mice was used for each carbon dioxide concentration tested. Of these, 25 were exposed to anoxia while inspiring the carbon dioxide, and the remaining 25 were exposed to anoxia alone. Three experiments were done each in duplicate. The first and the third of these each involved 10 mice exposed to anoxia while inspiring carbon dioxide and 10 control mice.

The concentrations of carbon dioxide inspired amounted to 2.5%, 5%, 7.5%, 10% or 20%.

The second stage of the experiment involved the effect of carbon dioxide on the survival of mice also anaesthetized with either thiopentone or halothane.

The length of survival of mice anaesthetized with thiopentone and simultaneously inspiring carbon dioxide was studied in the apparatus described above. The same apparatus was used for the halothane-anaesthetized mice, except that the evaporation chamber was replaced by a Dräger halothane evaporator.

The mice anaesthetized with thiopentone had this injected intravenously in an amount of 50 mg/kg body weight. When asleep the mice were placed in the bottles, which were then perfused for 10 minutes by a mixture containing 20% oxygen and 80% nitrogen. The mice were thereafter exposed to anoxia, the mixture conducted to the bottles being changed to 5% oxygen, 5% carbon dioxide and 90% nitrogen.

While the mice anaesthetized with halothane were being placed in the bottles, the latter were perfused with 20% oxygen and 80% nitrogen. When all the mice had been put in, the anaesthesia was begun with the above-mentioned mixture and 2% added halothane. The halothane concentration was reduced to 1% 2 minutes later. After the anaesthesia had been maintained with this concentration for 10 minutes, the mice were exposed to anoxia by inspiring a gas mixture consisting of 5% oxygen, 5% carbon dioxide, 1% halothane and nitrogen to 100%.

For the experiments with thiopentone we used 100 mice and for those with halothane 60 mice. In both experimental series half of the mice were anaesthetized and at the same time exposed to anoxia as well as to inspiration of carbon dioxide, the other half serving as controls, having only been exposed to anoxia.

Five experiments were done plus three duplicates experiments respectively, the survival period having been determined first for 10 anaesthetized mice and then for 10 controls.

All the percentages given are v/v.

A flow rate of 6 l/min was employed throughout.

The temperature was kept at between 28 and 32. In all the experiments.

The survival period, i.e. the time elapsed from the beginning of anoxia to the end of respiration, was measured for each mouse.

Results

The carbon dioxide concentrations used in the experiments, the average survival periods for mice exposed to carbon dioxide inspiration and anoxia, as well as for control mice exposed to anoxia alone, and the percentage prolongation of survival are shown in table 1

Inspiration of carbon dioxide at concentrations between 2.5 % and 10 % is seen to have prolonged the survival period. The prolongation was significant ($p < 0.01$) for the concentrations of 2.5–10 % Inspiration of 20 % carbon dioxide caused no prolongation of the survival period

The survival periods found for the thiopentone-anaesthetized and the halothane-anaesthetized mice after exposure to anoxia and simultaneous inspiration of carbon dioxide are shown in table 2. In the same table are also recorded the survival periods for the anoxic control mice, which had *neither* been anaesthetized *nor* inspired carbon dioxide. Finally the table contains the survival periods found in previous experiments (WILHJELM & ARNFRED 1965) for mice anaesthetized with thiopentone and with halothane and exposed at the same time to anoxia.

It is seen that the mice inspiring carbon dioxide while anaesthetized with thiopentone or halothane and exposed at the same time to anoxia survived longer than either the anoxic mice only inspiring carbon dioxide or the anoxic only mice anaesthetized with thiopentone or halothane

Inspiration of carbon dioxide at a concentration of 5 % by mice anaesthetized with either thiopentone or halothane caused a further in-

Table 1

Average survival periods in minutes, with s.e.m. values, for anoxic, but otherwise untreated controls, for anoxic mice exposed to inspiration of carbon dioxide and the percentage prolongations of the survival times.

50 mice were used for each carbon dioxide concentration, of which 25 served as controls. The oxygen concentration was 5 v/v % in all the experiments.

Anoxic, untreated control animals		Anoxic experimental animals exposed to inspiration of carbon dioxide			Percentage prolongation of survival time
Survival period in minutes	s.e.m.	Carbon dioxide concentration %	Survival period in minutes	s.e.m.	
2.9	0.14	2.5	4.4	0.32	50
2.9	0.18	5.0	6.9	0.21	137
2.7	0.06	7.5	5.4	0.26	98
2.8	0.10	10.0	4.1	0.[illegible]4	45
2.6	0.03	20.0	2.9	0.08	12

Table 2

Average survival periods in minuts with s.e.m values, for anoxic, but otherwise untreated controls, and for anoxic, thiopentone-anaesthetized and halothane-anaesthetized mice exposed at the same time to inspiration of 5 % carbon dioxide.

From previous studies, the average survival periods for anoxic, but otherwise untreated, controls and anoxic mice in thiopenton anaesthesia and halothane anaesthesia. Also the percentage prolongation of the survival periods.

Anoxic, untreated control animals		Anaesthetized, anoxic experimental animals			Percentage prolongation of survival period
Survival period in minutes	s.e.m.		Survival period in minutes	s.e.m.	
2.8	0.10	Thiopentone and carbon dioxide	14.1	1.67	400
2.9	0.07	Halothane and carbon dioxide	5.6	0.35	91
3.1	–	Thiopentone)	8.0	–	154
2.	–	Halothane*)	5.0	–	84

) Wilhjelm & Arnfred 1965

crease in the tolerance to anoxia beyond that produced by anaesthesia alone with one of the anaesthetics mentioned.

Discussion

Previous studies have shown that thiopentone (ARNFRED & SECHER 1962 WILHJELM & ARNFRED 1965) increases the tolerance of mice to anoxia.

Our investigation into the effect of carbon dioxide on the survival of anoxic mice showed that carbon dioxide increases the tolerance to anoxia when used at concentrations of from 2.5 to 10 % in the inspired air The prolongation is most pronounced at a carbon dioxide concentration of 5 % in the inspired air At this concentration the percentage prolongation of the survival period is of the same order as that seen after administration of thiopentone (WILHJELM & ARNFRED 1965) The failing prolongation of the survival after inspiration of 20 % carbon dioxide is presumably due partly to a depressive action on the vasomotor centre and partly to initiation of pulmonary oedema (POULSEN 1954).

In a study of the problems relating to inspiration of carbon dioxide at high altitudes it has been pointed out (FENN RAHN & OTIS 1946) that

inspiration of carbon dioxide will cause increased ventilation, which is accompanied by a rise in alveolar oxygen tension. Hyperventilation will bring about a rise in arterial oxygen saturation (HUSTON 1946 RAHN & OTIS 1947)

The increased tolerance to anoxia seen in mice after inspiration of carbon dioxide may thus be due either to raised arterial oxygen saturation or to increased cerebral blood flow or possibly both.

The survival period for anoxic mice exposed to inspiration of carbon dioxide was found to be of the same order as that for anoxic mice anaesthetized with thiopentone. This might, on a superficial view seem to bear out the hypothesis that the prolongation of the survival period caused by administering thiopentone to mice exposed to anoxia is due to accumulation of carbon dioxide with an ensuing increase in cerebral bloow flow and possibly also a rise in arterial oxygen tension.

However our experiments, in which thiopentone-anaesthetized mice were exposed to anoxia and at the same time to inspiration of 5 / carbon dioxide, showed an additional tolerance of mice to anoxia. Should the effect of thiopentone on the tolerance to anoxia depend on carbon dioxide retention, we would not expect thiopentone-anaesthetized mice exposed to anoxia and at the same time to inspiration of carbon dioxide to survive for a period approximately equal to the total survival period for anoxic mice after thiopentone administration alone and carbon dioxide inspiration alone.

Thus, there is much evidence to suggest that the increased tolerance to anoxia seen in mice after administration of thiopentone (WILHJELM & ARNFRED 1965) is mainly due to the depressive action of thiopentone on cerebral metabolism and not to accumulation of carbon dioxide.

In agreement with this explanation, it has been demonstrated (WILHJELM 1965) that hydroxydione (BAN) (= hydroxydion (WHO NFN) = viadril ®), which depresses cerebral metabolism to the same extent as do barbiturates, but is claimed not to affect the respiratory centre (GORDON, GUADAGNI, PICCHI & ADAMS 1956) increases the tolerance of mice to anoxia just as much as does thiopentone.

Inspiration of carbon dioxide will cause a rise in alveolar and arterial oxygen tension owing to hyperventilation. However the condition for inspired carbon dioxide to produce hyperventilation is that the respiratory centre is able to react to the increased arterial carbon dioxide tension. After administration of thiopentone to mice, the activity of the respiratory centre will be depressed. It therefore seems reasonable to assume that the appreciable prolongation of the survival of mice exposed to anoxia while anaesthetized with thiopentone and at the same time inspiring carbon dioxide is due to the depressant action of the barbiturate on

metabolism and to an increased arterial blood flow caused by the raised arterial carbon dioxide tension, and not to increased arterial oxygen tension

Halothane-anaesthetized mice exposed to anoxia will survive longer than non-anaesthetized mice exposed to the same degree of anoxia (WILHJELM & ARNFRED 1965) Simultaneous halothane-anaesthesia and inspiration of carbon dioxide (5 %) also cause prolongation of the survival of anoxic mice compared with that of non-anaesthetized control animals. The prolongation of the survival period is, however of the same order as that found for anoxic mice subjected to halothane anaesthesia alone. Thus, inspiration of carbon dioxide has no appreciable effect on the length of survival of anoxic mice under halothane anaesthesia.

Only a few investigations have been reported as to the effect of halothane on cerebral oxygen consumption and cerebral blood flow Studies by WOLLMAN (1963) seem, however to have shown that in halothane-anaesthetized human subjects cerebral oxygen consumption is only moderately reduced, whereas cerebral blood flow is increased. Wollman considers the reduction in cerebral oxygen consumption to depend in part on the fall of the body temperature taking place during halothane anaesthesia. In previous experiments as well as in those reported here the animals used were placed in a thermostat, and so are unlikely to have been subject to any great fall in body temperature

Accordingly the increased tolerance of halothane-anaesthetized mice to anoxia is probably mainly due to the increased cerebral blood flow attendant on the halothane anaesthesia.

In halothane-anaesthetized human subjects inspiration of carbon dioxide will cause an increase in cerebral blood flow independent of that due to the halothane anaesthesia (WOLLMAN 1963).

A further increase in cerebral blood flow need not, however be associated with any appreciable further prolonged survival of anoxic mice. In our study a 5 % concentration of carbon dioxide in the inspired air seemed to have the greatest effect in prolonging the survival of non-anaesthetized anoxic mice A continued rise in arterial carbon dioxide tension will cause a steady increase in cerebral blood flow in rhesus monkeys (REIVICH 1964) The same is likely to occur in mice. However inspiration of gas mixtures with rising concentrations of carbon dioxide will no longer have a corresponding effect in prolonging the survival of anoxic mice when the carbon dioxide concentration in the inspired air exceeds 5 %.

Thus, there seems to be a certain upper limit, at 5 % carbon dioxide in the inspired air beyond which an additional increase in cerebral blood flow has no effect on the tolerance of mice to anoxia.

Summary

Experiments have been conducted with different carbon dioxide concentrations in the inspired air with a view to studying their effects on the tolerance of mice to anoxia.

It appeared that inspiration of gas mixtures containing from 2.5 to 10 % carbon dioxide greatly prolonged the survival time of anoxic mice compared with that of control mice.

The prolongation of the survival period was most pronounced when the inspired air contained 5 % carbon dioxide

The effect of inspiring 5 % carbon dioxide on the tolerance to anoxia was also studied on mice anaesthetized with thiopentone and with halothane.

The thiopentone-anaesthetized anoxic mice inspiring a gas mixture containing 5 % carbon dioxide were found to be far more tolerant of anoxia than either non-anaesthetized anoxic control mice or halothane anaesthetized, anoxic mice. The further prolongation of survival time seen after admixture of carbon dioxide with the inspired air is attributed to its increasing effect on cerebral blood flow

Similar investigations into the effect of 5 % carbon dioxide inspired by halothane-anaesthetized anoxic mice gave the result that inspiration of carbon dioxide had no appreciable effect on the tolerance of these animals to anoxia, presumably because halothane anaesthesia as such increases the cerebral blood flow

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From the Department of Pharmacology University of Uppsala, Sweden
(Professor Ernst Bárány)

Oestrous Behaviour in Oestrogen Treated Ovariectomized Rats after Chlorpromazine alone or in Combination with Progesterone, Tetrabenazine or Reserpine

By

Bengt J Meyerson

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It is well-known that ovariectomized rats do not display oestrous behaviour when mounted by a male (ALLEN *et al* 1924 HEMMINGSEN 1932) Oestrous behaviour can, however be restored by treatment with oestrogen in combination with progesterone (BOLING & BLANDAU 1939 BEACH 1942).

The effects on oestrous behaviour of neuropharmacological substances which exert an effect on the central nervous monoaminergic mechanisms have been the subject of recent investigations.

It was found that the increase in monoamine levels due to monoamine oxidase inhibitors, decreased the oestrogen-progesterone activated heat response in ovariectomized rats. This inhibitory effect was especially evident after a selective increase in the cerebral serotonin (5-hydroxy tryptamine) content (MEYERSON 1964a & b).

The antagonistic relation between oestrous behaviour and cerebral monoamines was further indicated by the inhibitory effect of imipramine and related antidepressive substances (MEYERSON 1966)

Oestrous behaviour in ovariectomized rats requires both oestrogen and progesterone (BOLING & BLANDAU 1939 BEACH 1942). However it was demonstrated that progesterone, but not oestrogen treatment, could be replaced by the amine depletors, reserpine and tetrabenazine (MEYERSON 1964c).

Chlorpromazine brings about central nervous symptoms in the rat which are similar to some of those seen after reserpine treatment, e. g. catatonic rigidity decreased locomotor activity and diminished reaction to the environment. With both drugs it is also characteristic that, although obviously sedated, the animals can be "woken up" by being handled.

It is therefore of interest to investigate whether the similarities in central nervous effects of chlorpromazine and amine depletors such as reserpine or tetrabenazine are also found in the action of these drugs on oestrous behavior

Methods

The animals used were ovariectomized Sprague-Dawley rats weighing 200–320 gram. They were housed in airconditioned laboratories maintained at 22 24 During the observation period, i. e. 48–57 hours after the oestrogen injection, the animals were kept in special cages equiped with heating unit and thermostat which allowed the temperature to be regulated.

The day-night rhythm was reversed with light from 9 p. m. to 9 a. m. Oestrogen was injected at 8 a.m. and progesterone, tetrabenazine or reserpine 48 hours later Tests for oestrous behaviour were done between 10 a m. and 6 p.m.

A clear-cut copulatory response (lordosis reflex) on mounting by a vigorous male was *taken as an indication that the female was in heat. For a more detailed description of the* methods used see MEYERSON (1964 a b). Percentage inhibition was calculated as follows

$$100 - \frac{\text{Exptls \% in heat}}{\text{Controls \% in heat}} \times 100$$

The χ^2 test corrected for continuity or the FISHER exact probability test was used to test the significance of a difference between the effect of two treatments (see SIEGEL 1956, p. 96 and 104).

Recording of rectal temperature

Rectal temperatures were obtained by a Kipp & Zonen galvanometer (Microva A 14) supplied with a thermistor measuring device (Stantel thermistor type F 22). The thermistor was inserted to a depth of 40 mm. The individual data are the mean of two or three consecutive measurements.

Injected materials

Oestradiol benzoate and progesterone were dissolved in olive oil in volume of 0.20–0.30 ml. Chlorpromazine (hibernal ® Leo, ampoules 25 mg/ml) and reserpine (serpasil ® Ciba, ampoules 2.5 mg/ml) were diluted with distilled water Tetrabenazine-HCl (nitoman ® Hoffman-La Roche) pargyline-HCl (eutonyl ® Abbott) were dissolved in saline.

All injections were subcutaneous. The doses mentioned in the text and tables refer to the salts.

Results

The hypothermic and neuroleptic[1]) effects of chlorpromazine

The hypothermic effect of chlorpromazine in the rat is well established (HOFFMAN 1958 LE BLANC 1960 KOLLIAS & BULLARD 1964)

[1]) The term *neuroleptic* is used in the following to describe a state of decreased locomotor activity and exploratory behaviour and diminished awareness and reaction to environmental stimuli. It is characteristic that the neuroleptic animal is "woken up" by being handled, but on being left alone again, displays the above mentioned symptoms.

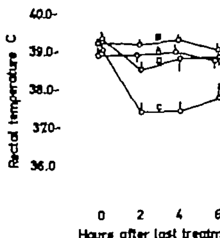


Fig. 1. The effect of chlorpromazine on rectal temperature in oestradiol benzoate (E) + progesterone treated ovariectomized rats.
Vertical lines = 2 × S. E.

Group	Treatment At 0 hrs.	Treatment At 48 hrs.	Treatment At 50 hrs.	Environmental temperature[1]) C°	N
A	none	none	saline	22 24	23
B	E. 10 µg/kg	progesterone 0.4 mg/animal	saline	23–24	23
C	E. 10 µg/kg	progesterone 0.4 mg/animal	chlorpromazine 5 mg/kg	22 24	24
D	E. 10 µg/kg	progesterone 0.4 mg/animal	chlorpromazine 5 mg/kg	30–32	23

[1]) From 48 hours onwards.

The normal rectal temperature in our ovariectomized rats was about 38.9° with only slight fluctuations according to the time of day at which the animals were observed for oestrous behaviour. The temperature level was not significantly influenced by the hormone treatment (Fig. 1 A & B).

A decrease in rectal temperature of about 1.5–2° was obtained at 2–6 hours after 5 mg/kg chlorpromazine when the animals were kept at 22–24° (Fig. 1 C). This decrease in the body temperature could be counteracted by increasing the environmental temperature to 30–32° (fig. 1 D).

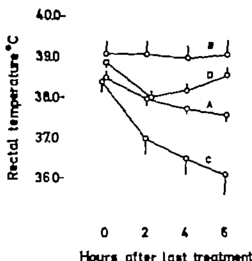


Fig. 2. The effect of chlorpromazine on rectal temperature in oestradiol benzoate (E) + tetrabenazine treated ovariectomized rats. Vertical lines = 2 × S.E.

Group	Treatment			Environmental temperature[1]) C°	N
	At 0 hrs.	At 48 hrs.	At 50 hrs.		
A	E. 10 µg/kg	tetrabenazine 40 mg/kg	saline	22–24	20
B	E. 10 µg/kg	tetrabenazine 40 mg/kg	saline	30–32	24
C	E. 10 µg/kg	tetrabenazine 40 mg/kg	hlorpromazine 5 mg/kg	22 24	20
D	E. 10 µg/kg	tetrabenazine 40 mg/kg	chlorpromazine 5 mg/kg	30–32	24

[1]) From 48 hours onwards

This was also found for 40 mg/kg tetrabenazine (Fig. 2, A & B). The hypothermic effect was evident 2 hours after the tetrabenazine injection (Fig. 2, A & C at 0 hours) However the hypothermic effects of a combination of tetrabenazine and chlorpromazine was only partially overcome by keeping the animals at 30–32 (Fig. 2, C & D)

In the investigation of the effect of chlorpromazine on oestrous behaviour the rectal temperature was measured during each run. The measurements were carried out after the last test for oestrous behavior and samples of 2 or 3 animals were also taken for the two other tests. If not otherwise stated in the text below these values were in full agreement with those shown in Fig. 1 and 2.

After 5 mg/kg of chlorpromazine, spontaneous activity was either absent or very slight when the animals were left undisturbed. However when handled in the tests for oestrous behaviour the animals "woke up" and moved about spontaneously although the exploratory behaviour was still clearly reduced. On being replaced in their own cages the animals locomotor activity decreased and they sat in a crouched position showing very little reaction to their environment.

There was still a neuroleptic effect with 1 mg/kg chlorpromazine, but the animals were moving around, spontaneously and when handled the motor activity and exploratory behaviour was almost the same as in animals treated only with hormones.

Ptosis was present to a variable extent in the animals treated with 5 mg/kg chlorpromazine, varying from a slight effect to in some animals, almost closed eyelids. The neuroleptic effect and the ptosis were the same in animals kept at 22–24 and at 30–32

Signs of catatonic rigidity were present after treatment with 5 mg/kg of chlorpromazine. When placed in an abnormal posture these animals remained in this posture for an obviously longer time then animals with only hormone treatment.

The symptoms described were fully developed 2 hours after treatment and lasted unchanged throughout the test period for oestrous behaviour

The neuroleptic effect, ptosis and catatonia were also seen in the reserpine or tetrabenazine treated animals. The effect 2–6 hours after 5–10 mg/kg chlorpromazine was roughly of the same order as those seen at 4–8 hours after injection of 40 mg/kg tetrabenazine or 1 mg/kg reserpine.

Chlorpromazine in combination with oestrogen alone

Chlorpromazine in doses of 1 5 or 10 mg/kg was administered 48 hours after 10 μg/kg oestradiol To prevent hypothermia the temperature in the cages in these experiments from 48 hours after the oestrogen injection was 32°

As is evident from Table 1 it was not possible to substitute chlorpromazine for the progesterone treatment. In comparison, 40 mg/kg of tetrabenazine, activated oestrous behaviour in 78 / and 1 mg/kg reserpine in 87 / (Table 2, controls) of the animals receiving this oestrogen treatment.

Chlorpromazine and progesterone tetrabenazine- or reserpine activated oestrous behaviour

Oestrous behaviour was activated by 10 μg/kg of oestradiol, followed 48 hours later by the injection of progesterone, tetrabenazine or reserpine.

Table 1

Heat response in ovariectomized rats treated with oestradiol in combination with chlorpromazine, progesterone or tetrabenazine. Oestradiol benzoate, 10 µg/kg, was injected at zero hour. The animals were kept at 30–32° from 48 hours.

Treatment at 48 hrs.		Hours after last treatment	Animals in Heat %	Number of animals tested	Number of rats pooled
Chlorpromazine	1 mg/kg	4–8	0	24	2
	5 mg/kg	4–8	0	35	3
	10 mg/kg	4–8	0	24	2
Progesterone	0.4 mg/animal	4–8	75	48	4
Tetrabenazine	40 mg/kg	4–8	78	23	2

Both the progesterone- and tetrabenazine-activated heat responses were significantly reduced by 10 mg/kg chlorpromazine (Fig. 3 and 4).

The effect of 5 mg/kg chlorpromazine was tested under two different experimental conditions, with an environmental temperature of 22°–24° and 30°–32° respectively. The heat response activated by progesterone was clearly reduced when the animals were kept at 22° while a very short-lasting effect of chlorpromazine was seen in the 30° experiment (Fig. 3).

The effect of 5 mg/kg chlorpromazine in the tetrabenazine experiment was smaller and about the same whether the environmental temperature was kept at 30–32° or 22–24°.

There was only a slight and transient inhibitory effect with 5 mg/kg chlorpromazine, on the oestrous behaviour after oestrogen-reserpine treatment (Table 2).

The combined treatment with both an amine depletor such as reserpine or tetrabenazine and chlorpromazine brought about an obvious neuroleptic effect. However, in spite of this, the female still reacted: she responded to the male mounting attempts with either a clearcut lordosis response or tried to defend herself.

Combined treatment with chlorpromazine and pargyline

The inhibitory effect on oestrous behaviour of the monoamine oxidase inhibitor pargyline has been established by previous investigations (MEYERSON 1964a & b).

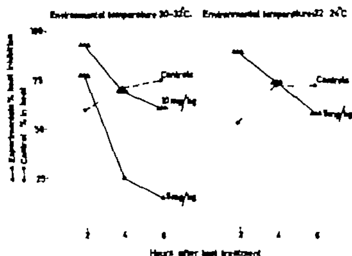


Fig. 3. The effect of chlorpromazine on oestrous behaviour in ovariectomized rats activated by progesterone in combination with oestradiol. Oestradiol benzoate, 10 µg/kg was injected at zero hour followed 48 hours later by 0.4 mg/animal progesterone. Controls had hormones alone.

Treatment At 50 hrs.	Environmental temperature[1]) C°	Number of Animals tested	Number of Runs pooled
Chlorpromazine 5 mg/kg	30–32	45	4
10 mg/kg	30–32	24	2
None (controls)	30–32	48	4
Chlorpromazine 5 mg/kg	22–24	47	4
None (controls)	22–24	36	3

[1]) From 48 hours onwards.

The number of triangles (Δ–ΔΔ) at the various points of the graph indicate the degree of statistical significance of the inhibitory effect, not the number of observations.

p	>0.05	<0.05 >0.01	<0.01
Symbol	Δ	ΔΔ	ΔΔΔ

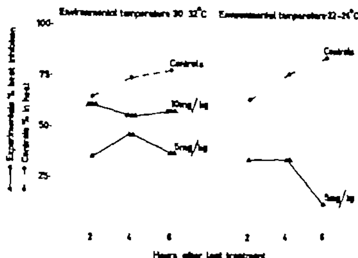


Fig. 4 The effect f chlorpromazine o oestrous behaviour in ovariectomized rats activated by tetrabenazine in combination with oestradiol. Oestradiol benzoate, 10 µg/kg was injected t zero hour followed 48 hours later by 40 mg/kg tetrabenazine. Controls had oestradiol and tetrabenazine alone.

Treatment At 50 hrs.	Environmental temperature[1]) C°	Number of	
		Animals tested	Runs pooled
Chlorpromazine 5 mg/kg	30–32	46	4
10 mg/kg	30–32	36	3
None (controls)	30–32	23	2
Chlorpromazine 5 mg/kg	22 24	48	4
None (controls)	22–24	33	3

[1]) From 48 hours onwards.

The number of triangles (Δ ΔΔ) at the various points of the graph indicate the degree of statistical significance of the inhibitory effect, not the number of observations.

P	>0.05	<0.05 >0.01	<0.01
Symbol	Δ	ΔΔ	ΔΔΔ

Table 2

The effect of chlorpromazine on oestrous behaviour in ovariectomized rats activated by reserpine in combination with oestradiol. Oestradiol benzoate, 10 μg/kg was injected at zero hour followed 48 hours later by 1 mg/kg reserpine. The animals were kept at 30–32° from 48 ho rs.

Treatment at 50 hrs.	Hours after last treatment	Animals in heat: Controls %	Animals in heat: Experimental % inhibition	P	Number of animals tested	Number of runs pooled
Chlorpromazine	2	55	55	>0.05		
5 mg/kg	4	87	43	<0.05	20–23	2
(controls had saline blanks)	6	87	13	>0.05		

Table 3

The effect of combined treatment with chlorpromazine and pargyline on oestrous behaviour in ovariectomized rats activated by oestradiol in combination with progesterone.
Oestradiol benzoate, 10 μg/kg, was injected at zero hour followed 48 hours later by 0.4 mg/animal progesterone. The animals were kept at 30–32 from 48 hours.

Treatment t 50 hrs.	Hours after last injection	Animals in heat: Controls %	Animals in heat: Experimental % inhibition	Number f animals tested	Number f runs pooled
A.					
Pargyline 25 mg/kg and	2	53	81[1])		
Chlorpromazine 5 mg/kg	4	66	97)	36	3
	6	75	90)		
(Controls had pargyline alone)					
B.					
Chlorpromazine 5 mg/kg	2	60	78		
(Controls had saline alone)	4	71	25	48–45	4
	6	75	15		

Treatment B values taken from F g. 3
Treatment A was significantly different from treatment B when compared 6 hours after the last injection. (P < 0.01).
[1]) Significantly different from controls (P < 0.01).

In the present experiments, pargyline was administered in a dose which did not reduce the heat response (Table 3 controls A vs. controls B).

When pargyline in a dose of 25 mg/kg was combined with 5 mg/kg chlorpromazine a reduction in the number of animals in heat was observed and thus was significantly greater than that seen after the use of chlorpromazine alone.

Discussion

Failure of chlorpromazine to elicit oestrous response response after oestrogen treatment

The normal oestrogen progesterone-activated oestrous behaviour in ovariectomized rats can be effectively activated by oestrogen in combination with reserpine or tetrabenazine, as is evident from the present and previous data (MEYERSON 1964c) It could be assumed that the central nervous amine depletion brings about an impaired monoaminergic transmission which could thus act as a substitute for the progesterone necessary for eliciting a response in these animals.

The present investigation shows, however that oestrous behaviour cannot be induced by chlorpromazine in the same way as reserpine or tetrabenazine Thus dissimilarity in central nervous effects of chlorpromazine and the amine depletors is in contrast to the induction of similar neuroleptic states by these drugs.

The mechanism of action of chlorpromazine on monoaminergic mechanisms is not clearly understood. Chlorpromazine does not seem to change the central monoamine levels quantitatively (COSTA, GARATTINI & VALZELLI 1960 GEY & PLETSCHER 1961), but has been shown to raise the brain levels of metabolites derived from catecholamines (CARLSSON & LINDQVIST 1963 GEY & PLETSCHER 1964 ANDÉN *et al.* 1964).

The increased formation of catecholamine metabolites after chlorpromazine treatment has been suggested by CARLSSON & LINDQVIST (1963) to be due to adrenergic receptor blockade. This would result in compensatory increased activity in catecholaminergic neurons with increased release of the amines and thus increased formation of catecholamine metabolites.

GEY & PLETSCHER (1962 & 1964) and PLETSCHER (1965) called attention to the inhibitory effect of chlorpromazine on transport across biological membranes, and considered the increased formation of phenylcarboxylic acids after chlorpromazine treatment to be at least partly due to impaired transport of the amines to the receptor and to storage sites.

It is now generally accepted that the neuroleptic effect of reserpine is brought about by depletion of central nervous monoamines. The evidence

suggests that it is mainly the impaired catecholaminergic transmission which is important (CARLSSON, LINDQVIST & MAGNUSSON 1957 EVERETT & WIBOAND 1962 CARLSSON 1964) The neuroleptic state after chlor promazine treatment is so similar to that induced by reserpine that a decreased central nervous adrenergic tone after chlorpromazine seems probable

It is clear from the present investigation that chlorpromazine failed to activate oestrous behaviour in oestrogen treated rats even in doses which induced a reserpine-like neuroleptic effect ranging from just slight (1 mg/kg) to a very obvious effect (10 mg/kg).

Previous work (MEYERSON 1964a & b) has demonstrated the presence of serotoninergic heat inhibitory pathways. It seems, therefore, that chlor promazine, although given in doses which impair adrenergic mechanisms, does not actually affect the serotoninergic neurons associated with the inhibition of oestrous behaviour

The biochemical data reported by ANDÉN *et al* (1964) that chlor promazine does not increase a metabolite of serotonin (5-hydroxy-indolacetic acid) in the rabbit corpus striatum in contrast to increased dopamine metabolites, also indicate that the effect of chlorpromazine on serotoninergic neurons might be different to its action on catecholaminer gic neurons.

Inhibitory effect of chlorpromazine

Chlorpromazine had an inhibitory effect on the progesterone- and tetrabenazine activated oestrous behaviour

The enhanced inhibitory effect when chlorpromazine was combined with the monoamine oxidase inhibitor pargyline, indicates that the inhibitory effect of chlorpromazine is due to an increased serotoninergic activity

The question of how chlorpromazine increases serotoninergic activity deserves further investigation. However as a working hypothesis, attention may be called to the fact that chlorpromazine has been shown to interfere with the axonal uptake of amines in adrenergic neurons (AXELROD, WHITBY & HERTTING 1961 MALMFORS 1965). If the compound acts similarly on central nervous serotoninergic neurons, this might explain the inhibitory effect observed in the present experiments with high doses of chlorpromazine, on progesterone or tetrabenazine activated oestrous behaviour The effect of chlorpromazine would then be analogous to the action of imipramine and related drugs which actively inhibit oestrous behaviour (MEYERSON 1966). (It is assumed that imipramine exerts this effect by preventing the serotonin uptake by the axon with subsequent increase of serotonin at the heat inhibitory receptors)

In the progesterone experiments, the effect of chlorpromazine was more evident in experiments in which the hypothermic effect of chlorpromazine was not prevented. Hypothermia might conceivably lead to an increase in the impairment of the membrane transport mechanisms. However the reason why hypothermia did not change the chlorpromazine effect in the tetrabenazine experiment remains to be explained.

Summary

Oestrous behaviour is normally activated in ovariectomized rats by progesterone after pre-treatment with oestrogen. Previous investigations have demonstrated that it is possible to replace progesterone by the amine depletors reserpine or tetrabenazine. The present data show that chlorpromazine administered in doses (1 5 or 10 mg/kg) which induce similar neuroleptic effects as the amine depletors, did not activate oestrous behaviour in oestrogen treated ovariectomized rats.

Chlorpromazine (10 mg/kg) had an inhibitory effect on progesterone- or tetrabenazine-activated oestrous behaviour In the progesterone but not in the tetrabenazine experiments, this inhibition was more evident if the hypothermic action of chlorpromazine was not prevented.

When chlorpromazine was combined with the monoamine oxidase inhibitor pargyline, an enhanced inhibitory effect on the progesterone activated oestrous behaviour was seen.

Evidence is presented in favour of the view that chlorpromazine acts in different ways on the central nervous serotoninergic and catecholaminergic mechanisms.

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From The Research Laboratories of AB Astra, Södertälje, Sweden

On Felypressin (Octapressin®) as an Adjunct to Lidocaine and Prilocaine – an Experimental Study in Animals

By

B. Åkerman

(Received March 4 1966)

The recently introduced synthetic polypeptide felypressin (= phenylalanine[2]- lysin[8]- vasopressin PLV 2, octapressin ®[1]) (BOISSONNAS & GUTTMANN 1960) has been found to possess a marked constrictor action on small blood vessels (BERDE, WEIDMANN & CERLETTI 1961 ALTURA, HERSHEY & ZWEIFACH 1965). It has been used clinically to reduce bleeding (CHALMERS 1961 HOCHULI 1962)

The predominantly peripheral action of felypressin suggested that this vasoconstrictor might be of value as an alternative to adrenaline in local anaesthetic solutions An enhancement of the local anaesthesia was generally observed when felypressin was used in combination with lidocaine (HOCHULI 1962 KLINGENSTRÖM & WESTERMARK 1963), with oxyprocaine (MISSURA & WEDER 1962) and with procaine (DE GRENUS 1963 BOLLOBÁS 1963). Certain side-effects were, however reported with the oxyprocaine and procaine solutions, probably depending on the rather large volumes and the comparatively large amounts of felypressin used.

It is known that the effect of an added vasoconstrictor varies with the type and the concentration of the local anaesthetic agent. This has been demonstrated with regard to the enhancement of the anaesthetic effect (LIDOUBNA 1960 ÅSTRÖM & PERSSON 1965) as well as to the toxicity of these agents (HOLLER 1952 AVANT & WEATHERBY 1960 ÅSTRÖM, PERSSON & ÖRTENGREN 1964) With this background it was considered desirable to study the influence of the polypeptide felypressin on the anaesthetic and toxic properties of some local anaesthetics In the study reported here, the action of felypressin in combination with lidocaine and prilocaine (citanest ®) has been compared with that of adrenaline in some animal experiments.

[1] Sandoz Ltd., Basle, Switzerland.

Material and Methods

Absorption studies. Female Sprague Dawley rats (175–200 g) were used for two series of experiments. In *one series* the rate of clearance of ^{22}N was studied after i. m. injection of 0.9 % ^{22}Na-chloride solution (^{22}Na specific activity = 1 7 μC/ml) according to the technique of ÅSTRÖM, PERSSON & TALČ (unpublished). The effects of addition of 0.01 0.1 and 0.5 IU/ml felypressin corresponding to 0.18, 1 8 and 9.0 μg/ml of the polypeptide and 1 100000 (10 μg/ml) adrenaline were compared. One group of animals was injected with ^{22}NaCl alone as control. 0.. ml of the test solution was injected into the sciatic nerve pocket of one of the hind limbs of the rat. Each solution was tested in 20 animals. Half the number of animals in each series was sacrificed 10 minutes after injection and the others 30 minutes after injection. The hind limbs were separated from the body after removal of skin and fat and disintegrated in 10 ml fuming nitric acid. The content is diluted with distilled water to a final volume of 50 ml of which a 5 ml sample was taken for determination of the radioactivity

In *a second series* the rate of absorption of tritium labelled (YAVORSKY & GORIN 1962) lidocaine and prilocaine was compared after addition of 0.01 0.1 and 0 5 IU/ml felypressin and 1:100000 adrenaline to the solutions. 0.05 ml of each solution (1.0 %) was injected into th tip of the tongue in groups of 10 animals. The rats were sacrificed 8 minutes after the injection. The tongues were rapidly removed, weighed and homogenized in 5 ml 5.0 % trichloroacetic acid (TCA) solution. The homogenates were made lkaline with 10 N sodium hydroxide to a pH above 10.0. After addition of 15 ml toluene the samples were mechanically shaken for 20 minutes. 5 ml of the toluene extract were added to 10 ml of the scintillation liquid (3 g 3 4-diphenyloxazole, 0.1 g β-bis/2-(phenyloxazolyl)/benzene in 1 litre of mixture containing 200 ml ethanol and 800 ml toluene). The samples were then measured f radioactivity in a Packard Tricarb Autoanalyzer Known amounts of the labelled compounds were added to tongue homogenates. These samples were used as standards after preparation and treatment, in the same way as the test samples. A recovery of 90–100 % of the labelled local anaesthetic agents was btained in these experiments.

The influence on the *local anaesthetic action* was studied by intracutaneous heals in guinea-pigs according to BÜLBRING & WAJDA (1945), slightly modified, and on sciatic nerve blocks in rats (TRUANT 1958). The following solutions were compared in the *intracutaneous wheal* tests (1) 0.125 % lidocaine or prilocaine (2) 0.125 % lidocaine or prilocaine ith felypressin, 0.01–0 5 IU/ml (0.18–9 0 μg/ml) (3) 0.125 % lidocaine or prilocaine with adrenaline, 1 300000 (3 33 μg/ml) and 1 100000 (10 μg/ml) Each felypressin concentration was tested gainst the plain solutions and those with adrenaline. Each felypressin concentration was studied in nine guinea-pigs, two wheals being produced on each animal. The anaesthetic solutions with and witho t drenaline were injected as two new wheals in the *same animals, each of the solutions thus being tested on 3 animals.* The solutions ith felypressin were pplied on the first day and the plain or adrenaline containing solutions 24 hours later or ice versa. 0.25 ml of the solutio was injected intracutaneously on the shaven backs of the animals. The anaesthesia was tested by pin-pricking six times at different places within the wheal at regular intervals f five minutes and observing the skin contraction around the injected area.

In the *sciatic nerve block* experiments, female Sprague Dawley rats (160–190 g) ere injected in the sciatic nerve pocket t mid thigh level. The following solutions were compared: (1) 2.0 % lidocaine or prilocaine (2) 2.0 % lidocaine or prilocaine ith felypressin 0.01–0.5 IU/ml (0.18–9 0 μg/ml) (3) 2.0 % lidocaine or prilocaine with adrenaline 1 300000 (3.33 μg/ml), 1 200000 (5.0 μg/ml) and 1 100000 (10 0 μg/ml). Each felypressin concentration was tested in 24 rats. 0.2 ml of the solution was injected in one of the legs of each

animal. The contralateral thigh of the same animal was injected with the plain or adrenaline containing solutions, each solution being tested in six animals. The two experiments were carried out with a 4 hour interval. The order of the experiments was randomized. Only complete motor paralysis was recorded as a block. Incidence, latent period and duration of anaesthesia were determined.

The *acute toxicity* (subcutaneous and intravenous) of lidocaine and prilocaine in combination with felypressin was determined in male albino mice weighing about 20 g. The felypressin concentrations studied were 0.1 and 0.5 IU/ml (1.8 and 9.0 µg/ml) and for adrenaline 1 200000 and 1:100000 (5 and 10 µg/ml). The concentrations of the local anaesthetic agents used were 0.1 0.2 and 2.0 % respectively The injected volume did not exceed 0.5 ml. The LD50 values of the solutions were estimated by the graphical method of MILLER & TAINTER (1944). The significance of the differences in toxicity between the different solutions was calculated according to the Student's t-test.

The *local irritation* of 2.0 % lidocaine and prilocaine solutions with 0.1 to 1.0 IU/ml (1.8 to 18.0 µg/ml) felypressin was compared to that of the same local anaesthetic solutions with adrenaline 1 80000 (12.5 µg/ml) on rabbit ears. The reactions after injection of 0.1 ml of the solutions between the dermal layers of the ears were observed for seven days (WIEDLING 1952).

The *respiratory and circulatory effects* of felypressin in various combinations with lidocaine and prilocaine were compared to those of adrenaline containing solutions in pentobarbitone (35 mg/kg i. p.) anaesthetized cats weighing 2.4–2.6 kg. Ten mg of the local anaesthetics in 2.0 % solutions were injected into the femoral vein over period of 30 seconds. Respiration and systemic blood pressure (carotid artery) was recorded on a direct recording Grass oscillograph by strain gauge pressure transducer

Lidocaine and prilocaine were used as the hydrochlorides dissolved in 0.9 % saline. The pH of the sterile solutions was 4.8–5.2 except in the toxicity studies where it was adjusted to 6.5. Felypressin was added from a concentrate (Sandoz, Basle) of the following composition synthetic PLV 2, 25 ± 3 IU (1 IU corresponded to 0.018 mg PLV 2) sodium acetate, 2.0 mg glacial acetic acid, 1.0 mg sodium chloride, 0.3 mg trichlorisobutylalcohol, 2.0 mg and distilled water ad 1.0 ml, pH 4.5

Results

Absorption studies 0.5 IU/ml felypressin retarded the ^{22}Na clearance from the hind limb of the rat to a greater extent than 0.1 and 0.01 IU/ml (Fig. 1). There were no significant differences in activity between felypressin 0.1 or 0.5 IU/ml and adrenaline 1 100000 ten minutes after the injection, while 0.01 IU/ml felypressin was less active than adrenaline 1 100000. The figures obtained 30 minutes after injection indicated that the effect of felypressin was of a shorter duration than that of adrenaline.

The assumption that felypressin, like adrenaline, retards the transport of lidocaine and prilocaine away from an injection site was tested in another series of experiments in rats. The plain solutions rapidly disappeared after injection into the tongue (Table 1) 0.5 IU/ml felypressin was more effective than adrenaline (1 100000) in retarding the absorption of lidocaine and prilocaine in this highly vascularized area. The effect of 0.01 IU/ml felypressin was not significantly different from that of adrena-

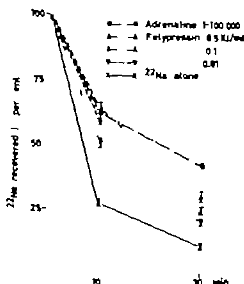


Fig. 1 Clearance of ^{22}N from the hind limb of the rat. Injection of 0 2 ml of 0.9 % ^{22}NaCl with and without adrenaline or felypressin (n = 10).

Table 1

Effects of adrenaline and felypressin on the bsorption of lidocaine and prilocaine from th rat to gue. Remaining amount (per cent of injected dose) in the tongues eight minutes after injection of 0.5 µg 3H-labelled agents in groups of ten animals.

Vasoconstrictor	Conc. µg/ml	Conc. IU/ml	Lidocaine	Significance of difference adrenaline-felypressin p	Prilocaine	Significance of difference adrenaline felypressin p
Adrenaline	10.0		40 0 ± 2.2		41.2 ± 2.5	
Felypressin	0.18	0.01	38 3 ± 2.6	p > 0.05	37.8 ± 2.9	p > 0.05
	1.8	0.1	46.6 ± 3.3	p > 0.05	51.2 ± 2.8	0 05 > p > 0.01
	9.0	0 5	63 4 ± [illegible]1	p < 0.001	54.2 ± 3.[illegible]	0 01 > p > 0 001
Plain solutions			11 6 ± 2.3		16.3 ± 1 1	

line 1 100 000 These two series of experiments indicated that felypressin affected the initial rate of absorption of injected solutions to a higher extent than adrenaline. 1 8 µg/ml (0.1 IU/ml) felypressin, for instance, was at least as active as 10 µg/ml adrenaline shortly after the injection. On the other hand, the ^{22}Na-clearance studies showed that the duration of action was shorter with felypressin than with adrenaline.

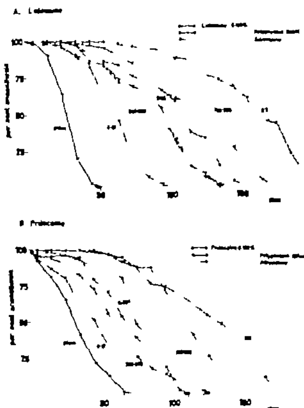


Fig. 2. Intracutaneous wheals in guinea-pigs. The effect of 0.25 ml of 0.125 % (A) lidocaine or (B) prilocaine with and without felypressin and adrenaline expressed as per cent successful anaesthesia at different times after injection. Each point represents the mean of 108 pin-pricks within 18 wheals (felypressin added) or 144 pin-pricks within 24 wheals (adrenaline added o plain solutions).

Effect on the local anaesthetic action. Felypressin was found to prolong the duration of local anaesthesia in wheal infiltration tests in guinea-pigs (fig. 2). 0.05 IU/ml (0.9 μg/ml) felypressin was at least as effective as adrenaline 1 300000 (3.33 μg/ml) 0 1 IU/ml felypressin in combination with prilocaine (Fig. 2B), but not with lidocaine (Fig. 2A), was more effective than adrenaline 1 100000 The net increase in duration was small at higher concentrations of felypressin. Fig. 2 also shows that normal response to stimuli returned somewhat faster with prilocaine felypressin than with corresponding lidocaine felypressin solutions. The differences in effect of the two agents were larger for the solutions with adrenaline. Lidocaine-adrenaline was longer acting than corresponding

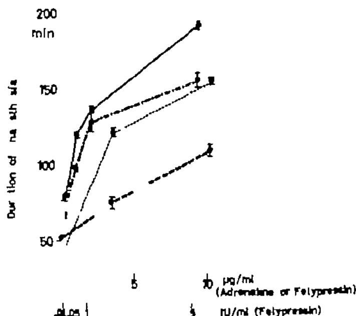


Fig. 3 Intracutaneous wheals in guinea-pigs. Time for return of normal response to stimuli (>90% of the responses positive) after injection of 0.25 ml of 0.125% lidocaine or prilocaine with felypressin or adrenaline. Mean of 16 wheals per local anaesthetic and concentration of felypressin and mean of 24 wheals per agent and concentration of adrenaline or plain solution. Symbols ———— = lidocaine + felypressin; — — = prilocaine + felypressin = lidocaine + adrenaline ——— = prilocaine + adrenaline.

prilocaine-adrenaline solutions. This difference is illustrated in Fig. 3 in which the time for complete recovery (>90% positive responses) is plotted against concentrations of the vasoconstrictors.

Felypressin addition did not influence the short onset times and the high frequencies of anaesthesia observed with lidocaine and prilocaine in sciatic nerve block in rats. Complete motor paralysis was obtained in all animals within two minutes. The lowest concentration tested, 0.01 IU/ml, prolonged the duration with both compounds, to at least the same degree as adrenaline 1:300000 (Fig. 4). 0.05 IU/ml had the same effect as adrenaline 1:100000. However, a further increase in felypressin addition did not cause any marked enhancement of the effect. The results from experiments with solutions that had been stored at +4° eight months after their first use, did not significantly deviate from those obtained in the first series of experiments.

Acute toxicity. The acute subcutaneous and intravenous toxicities of some lidocaine and prilocaine solutions with felypressin or adrenaline were compared in mice. Table 2 shows that the felypressin (0.1 and 0.5 IU/ml) containing solutions of both agents were less toxic than the plain

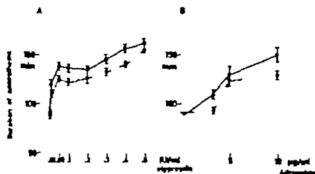


Fig. 4. Sciatic nerve block in rats. Duration of anaesthesia produced by 0.2 ml of 2.0% lidocaine or prilocaine in combinations with felypressin (A) and adrenaline (B). Mean of 24 rats per local anaesthetic agent and felypressin concentration and mean of 44 rats per agent and adrenaline concentration or plain solution. Symbols ●——● = lidocaine ○——○ = prilocaine.

Table 2

Effect of felypressin and adrenaline on the acute subcutaneous toxicity (mice) of lidocaine and prilocaine. LD50 ± s.e. (n = 60) calculated by the method of MILLER & TAINTER (1944).

Local anaesthetic	Felypressin conc. IU/ml	LD50 ± s.e. LA mg/kg	significance of difference p	Adrenaline conc. $\times 10^{-3}$	LD50 ± s.e. LA mg/kg	significance of difference p
Lidocaine	0	238 ± 11		0	238 ± 11	
	0.1	311 ± 15	$0.01 > p > 0.001$	1:200	212 ± 11	$p > 0.05$
	0.5	288 ± 15	$0.05 > p > 0.01$	1:100	196 ± 7	$0.01 > p > 0.001$
Prilocaine	0	637 ± 18		0	637 ± 18	
	0.1	770 ± 19	$p < 0.001$	1:200	446 ± 16	$p < 0.001$
	0.5	719 ± 12	$0.05 > p > 0.01$	1:100	386 ± 14	$p < 0.001$

solutions after subcutaneous administration. Adrenaline (1:200000 and 1:100000), on the other hand, increased the acute subcutaneous toxicity of lidocaine and prilocaine in mice. The intravenous acute toxicity was not increased by felypressin while the adrenaline containing solutions were more toxic than the plain solutions (Table 3).

Local irritation. The local tissue reaction on rabbit ears after injections of lidocaine and prilocaine solutions with felypressin and adrenaline respectively differed somewhat. A pale ischaemic zone which gradually disappeared was observed after injection of the solutions with felypressin

Table 3

Effect of felypressin and adrenaline on the acute *intravenous* toxicity (mice) of lidocaine and prilocaine. LD50 ± s.e. (n = 60) calculated according to MILLER & TAINTER (1944).

Local anaesthetic	Felypressin conc. IU/ml	LD50 ± s.e. LA mg/kg	significance of difference *p*	Adrenaline conc. $\times 10^{-3}$	LD50 ± s.e. LA mg/kg	significance of difference *p*
Lidocaine	0	17.8±0.9		0	17.8±0.9	
	0.1	26.7±1.1	p<0.001	1:200	13.6±1.0	0.01>p>0.001
	0.5	15.8±1.2	p>0.05	1:100	10.4±1.0	p<0.001
Prilocaine	0	35.0±1.7		0	35.0±1.7	
	0.1	34.3±2.5	p>0.05	1:200	23.6±1.7	0.01>p>0.001
	0.5	34.6±0.6	p>0.05	1:100	18.9±0.8	p<0.001

(0.1 to 1.0 IU/ml). Addition of adrenaline (1:80000) initially produced faint pallor at the injection site that soon was followed by slight hyperaemia. None of the solutions gave any signs of persistent tissue damage and the very weak to weak effect disappeared within a few days.

Respiratory and circulatory effects in cats. Felypressin by itself in concentrations below 0.1 IU/ml administered in rapid intravenous injection in 0.5 ml isotonic sodium chloride solution did not affect circulation or respiration. 0.5 IU/ml caused a moderate and prolonged increase in the blood pressure (max. + 16 mm Hg). The respiration was unaffected.

The regularly observed transitory blood pressure fall in the carotid artery following lidocaine administration was abolished when 0.5 *IU/ml* felypressin was added to the test solution (Fig. 5A). A slight initial rise was observed with increasing felypressin concentrations (*1.0 IU/ml*). In contrast to felypressin, adrenaline (1:100000 and 1:200000) did not counteract the immediate fall in blood pressure caused by lidocaine. Addition of adrenaline also gave a marked secondary pressor effect before the return to normal. The lidocaine felypressin combinations did not influence respiration more than the plain lidocaine solutions.

The fall in blood pressure observed with prilocaine is not so marked as that after lidocaine administration (WIEDLING 1960, ÅSTRÖM & PERSSON 1961). In these experiments a pressor effect was observed with somewhat lower felypressin concentrations when combined with prilocaine than when added to lidocaine (Fig. 5B). Respiration was unaffected after the doses of felypressin studied.

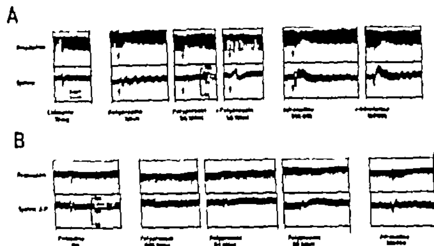


Fig. 5. Cat (2.5 kg). Respiratory and circulatory effects of lidocaine (A) and prilocaine (B) with and without felypressin or adrenaline. Intravenous injections of 0.5 ml of 2.0% solutions with 30 minutes intervals between each injection.

Discussion

These experiments have shown that felypressin has a marked effect on the absorption rate from the site of injection. The transport of ^{22}Na away from the rat thigh and of lidocaine and prilocaine away from the rat tongue was retarded by low concentrations of the polypeptide.

A number of lidocaine-felypressin and prilocaine-felypressin combinations were tested for local anaesthetic effect by infiltration on the back of guinea pigs and by sciatic nerve block in rats. The felypressin content was adjusted on the basis of the results obtained from the clearance studies. It was shown that, compared on a weight basis, felypressin prolonged the duration of the local anaesthesia more than adrenaline. 0.9 µg/ml of the polypeptide for instance was about as active as 3.33–5.0 µg/ml of the catecholamine. These findings correspond to those obtained by BERDE, SCHALCH & DOEPFNER (1964) who compared the effects of felypressin and adrenaline added to procaine. The duration of the infiltration anaesthesia was enhanced with increasing felypressin concentrations as was found with adrenaline. The gain was, however, small when the felypressin content was increased above 0.1 IU/ml. A rough dose activity relationship for felypressin was also obtained in the nerve block experiments. Increase of the polypeptide amount in the prilocaine solutions from 0.05 IU/ml to 0.1 IU/ml and in the lidocaine solutions from 0.05 to 0.1 and 0.2 IU/ml tended, however, to decrease the duration of the anaesthesia. Although the differences were small they are probably relevant, since significant decrease of the duration by increasing the fely-

pressin concentration from 0.05–0.5 IU/ml has been obtained in dental studies with prilocaine-felypressin and lidocaine-felypressin solutions (BERLING 1966). The reason for this unexpected effect has not yet been studied. It may be correlated with the mechanism of action of felypressin which has been shown to differ from that of adrenaline (CERLETTI, WEBER & WEIDMANN 1963, ZWEIFACH 1963, ALTURA, HERSHEY & ZWEIFACH 1965).

The results obtained in this study regarding the effectiveness of felypressin cannot be expected to be generally applicable. The type as well as the concentration of the local anaesthetic agent will influence the results. The interaction between local anaesthetic agents and adrenaline is affected by such factors (ÅSTRÖM & PERSSON 1965). The site of administration is undoubtly another factor of importance. This is illustrated by the differences in effect following injection into the tongue and thigh of rats. 0.01 IU/ml felypressin was as effective as adrenaline 1:100000 in retarding the absorption rate from the rat tongue shortly after injection. In the thigh this felypressin concentration was inferior to adrenaline 1:100000 at 10 and still more at 30 minutes after injection. However, the duration of the nerve block was longer with felypressin than with the adrenaline combinations. The marked initial vasoconstrictor effect of felypressin thus seems to be important. This is also probably reflected by the toxicity studies in mice where a low felypressin concentration decreased the lethal doses of lidocaine and prilocaine considerably on subcutaneous administration. Preferably, these subjects should be further analyzed with regard to the new vasoconstrictor felypressin.

The results from this investigation would seem to justify further evaluation of felypressin as an adjunct to local anaesthetic agents. Small amounts of felypressin delayed the absorption of lidocaine and prilocaine and prolonged the duration of the local anaesthesia of these two agents. The felypressin combinations were less toxic than the solutions with adrenaline. This was true even after intravenous injection. Sufficient enhancement of the duration of local anaesthesia was obtained when about 0.05 IU/ml felypressin was added to either lidocaine or prilocaine solutions. With such a low felypressin concentration the risk for side-effects similar to those reported with procaine-felypressin (DE GREGORIO 1963) and oxyprocaine-felypressin (MIESTURA & WEDER 1962) should be minimal. From a practical point of view it is also important that the felypressin solutions have proved to be very stable. No diminution of the effect could be observed with solutions that had been stored at +4° (pH 4.8) for 8 months. When stored at room temperature (+25°) for 20 months a solution containing 0.27 IU/ml (pH 4.0) lost only 6 % of the activity (DAHLINDER, personal communication).

Summary

A powerful vasoconstrictor effect has been ascribed to the synthetic polypeptide felypressin (octapressin ®) These findings suggested that felypressin might be useful as a supplement to solutions of local anaesthetic agents.

Felypressin retarded the absorption of lidocaine and prilocaine and prolonged the duration of local anaesthesia of both agents more than adrenaline when compared on a weight basis. 0.05 IU/ml (0.9 µg/ml) of felypressin was about as active as adrenaline 1 300000 to 1 200000 (3 33 to 5.0 µg/ml)

In contrast to adrenaline, felypressin decreased the acute subcutaneous toxicity of both agents in mice and the intravenous toxicity of both agents was not increased. The transitory falls in blood pressure observed with lidocaine and prilocaine were abolished by low felypressin concentrations. High concentrations produced a moderate and prolonged pressor rise The felypressin containing solutions were not locally irritating and were very stable.

The results of this investigation would seem to justify further evaluation of solutions of local anaesthetic agents with felypressin as a vasoconstrictor

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From the Research Laboratories of AB Astra, Södertälje, Sweden

Studies on the Absorption, Distribution and Metabolism of Labelled Prilocaine and Lidocaine in some Animal Species

By

B. Åkerman, A. Åström, S. Ross and A. Telc[1]

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The local anaesthetic agent prilocaine (α propylamino-2 methyl-propionanilide, citanest ®), synthesized by LÖFGREN & TEGNÉR (1960), has been found to have about the same potency as lidocaine in several pharmacological *in vivo* tests (ÅSTRÖM & PERSSON 1961) as well as in various clinical trials (ERIKSSON & GORDH 1959 BERLING & BJÖRN 1960 CRAWFORD 1964). The agent is, however only about half as toxic as lidocaine in *i v* mice (WIEDLING 1960) This difference in toxicity is particularly marked when the two agents are compared by slow intravenous infusions in mice or by repeated intravenous injections in rabbits (ÅSTRÖM & PERSSON 1961) In man prilocaine is also less toxic than lidocaine when given intravenously (ENGLESSON *et al* 1962)

A local anaesthetic agent with an improved ratio between effectiveness and toxicity is of interest clinically as well as from a pharmacological point of view A study of the factors determining the toxicity of such an agent would be of particular interest with regard to the question of whether or not the toxicity of local anaesthetic agents is directly correlated to their local anaesthetic potency

In the present study various factors determining the action of and the difference in toxicity between prilocaine and lidocaine have been analysed in some detail. The rate of absorption and distribution in different tissues, elimination and rate of metabolism *in vitro* and *in vivo* have been investigated in some laboratory animals.

[1] Present address Packard Instrument International, Zürich 1 Switzerland.

Methods

The compounds studied, lidocaine (xylocaine ®) and prilocaine (citanest ®), were used as the hydrochlorides, dissolved in 0.9 % NaCl solution, and the pH adjusted to 6.5. ^{14}C labelling on the carbonyl group of lidocaine and prilocaine was performed according to TEGNÉR & DOMEIJ (1962). For some of the studies the same compounds were tritiated according to YAVORSKY & GORIN (1962). By this procedure the labelling takes place on the benzene nucleus.

The rate of absorption was studied with ^{14}C labelled and tritiated compounds in the tongues of female Sprague-Dawley rats (180–200 g). In a first series of 48 rats 0.05 ml of 1.0 % solutions were injected in the tip of the tongues after insertion of the injection needle for about 2 mm. In a second series of 24 animals, 2.0 % ^{3}H-lidocaine and ^{14}C-prilocaine solutions were mixed and 0.05 ml of the mixture injected as in the first series. At different time intervals after injection the animals were decapitated and the tongues rapidly removed, weighed and stored overnight at −17°. Extractions and determinations were performed as described below.

The rate of absorption of the two agents from the thigh was also studied in rats by analysis of the amount remaining at different times after the injection of ^{14}C-labelled compounds in the sciatic nerve pocket. Skin and thigh bone were removed before homogenization and extraction.

The distribution in various tissues was studied in rats (same strain as above) following the injection of the agents by different routes. In a *first series* of experiments, a dose of 10 mg/kg body weight (1.0 % solution of either ^{14}C-prilocaine or ^{14}C-lidocaine) was injected intramuscularly in the sciatic nerve pocket of rats. The animals were killed 2 to 120 min. after the injection, the organs rapidly removed, weighed, frozen and later assayed as described below. The blood was analyzed immediately. In a *second series* of experiments the animals were given a mixture of 5 mg/kg ^{14}C-prilocaine plus 5 mg/kg ^{3}H-lidocaine intraperitoneally, intramuscularly or subcutaneously. The concentration of the agents in various tissues was then determined as in the first series. In a *third series* the concentration of ^{14}C labelled lidocaine and prilocaine in the blood and the brains of rats was determined at the time when signs of toxicity (convulsions) had developed to the same degree. In mice a similar study was made and in these animals the analysis was also made at the time when the convulsions subsided. The injections were made intraperitoneally after a preliminary series of tests had been carried out to determine doses which produced similar degrees of toxicity. In a *fourth series* the local distribution of the agents was studied after the injection of 0.2 ml of 1.0 % solutions into the sciatic nerve pocket of rats at mid thigh level and the concentration in the nerve and surrounding muscles determined at the time when motor block subsided.

The elimination of prilocaine in the urine and the faeces after intraperitoneal injection of 20 mg/kg was estimated with the ^{14}C-labelled compound in 12 rats placed in metabolic cages for 24 hours. The animals were given water and food (pellets) *ad libitum*. The urine was collected in 10 ml of ethanol at 2 and 6 hours after injection. After filtration one part of the urine was taken for determination of the total amount of radioactivity as described below. Another part of the urine was extracted and analysed for the content of ^{14}C prilocaine as described below. The faeces were treated in the same way after homogenization.

The metabolism in vivo was studied in mice (DSS, 18–22 g). ^{14}C-labelled lidocaine and prilocaine solutions (1.0 %) were injected intraperitoneally. At different times afterwards the animals were killed and immediately homogenized. Extraction of the homogenates was carried out as described below. For the *in vitro* studies liver, kidney or lung homogenates

as well as slices from different animal species were incubated with the labelled compounds for 10–60 min. at 37° The homogenates were prepared in 10 volumes of ice cold 0.05 N tris buffer at pH 8.5. Half a ml of the homogenates was incubated with 48 μg of ^3H or ^{14}C-labelled prilocaine and lidocaine in a final volume f 1.0 ml. The same amounts of the local anaesthetic agents were also added to 250 mg of the slices in 3 ml Krebs-Henseleit buffer (pH 7.4) incubated as above in an tmosphere of 93.5 % O_2 and 6.5 % CO_2

The extraction procedure was essentially the same as that used by SUNG & TRUANT (1954). The homogenization of the tongue and other tissues was carried out in 5 volumes of 5 % trichloroacetic acid (TCA) solution. After alkalinization with 10 N-N OH to a pH above 10, extraction of lidocaine and prilocaine was carried out with 15 ml toluene by mechanical shaking for 20 min. Five ml of the toluene extract was added to 10 ml scintillation fluid (3 g 3,4-diphenyloxazole, PPO, 0.1 g β-bis-[2-(phenyloxazolyl)] benzene, POPOP in a mixture containing 200 ml ethanol and 800 ml toluene). *Measurement of the radioactivity* was made by means of liquid scintillation counter (Packard Tricarb). Standards were made by adding known amounts of the labelled compounds to non-incubated tissue homogenates prepared and treated in the same way as the test samples. The recoveries of the local anaesthetic gents from homogenized tissues were better than 90 %.

The incubated homogenates and slices used for the metabolic studies were treated with 1.0 ml 10 % TCA at the end of the incubation period. After adding 5 ml borate buffer pH 10, the ^{14}C-prilocaine samples were shaken for 20 min. with 20 ml heptane and the ^{14}C-lidocaine samples with the same volume of toluene.

The tritiated compounds were all extracted with heptane at pH 10. In this case the radioactivity of the extract could partly be derived from aromatic metabolites, e g o-toluidine and 2,6-xylidine. To study this possibility part f the heptane extract was shaken with an equal volume of MACILVAINE (1921) buffer at pH 4.0. At this pH, intact prilocaine and lidocaine was transferred almost quantitatively to the water phase while added o-toluidine and 2,6-xylidine were found to remain in the heptane phase to an extent of 32 and 87 % respectively. This extraction procedure was used only to get rough estimate of the aromatic breakdown products formed. Quantitative determination as well as definite chemical identification of the aromatic products formed were not made in this study

The samples were prepared for measurement of radioactivity by adding 10 ml scintillation fluid (ethanol, POP and POPOP in toluene as above) to 2.5 ml of the heptane and toluene extracts.

For paper chromatographic separation of break-down products formed after incubation of homogenates or slices, the followi g solvents were used

I t-butanol 6, acetic acid 1 water 3 /v

II n-butanol saturated with 25 % acetic acid

III methylethylketone 80, n-butanol 40, acetic acid 1 water 15 /v

Results

The *absorption* of lidocaine and prilocaine was studied following the injection into the tongue of rats, by analyzing the amounts of drug retained in this tissue. The results (table 1 A) showed that at 2, 4 and 8 min. after the injection more prilocaine than lidocaine remained in the tongues.

It has previously been observed (ÅSTRÖM & PERSSON 1961) that prilocaine seems to enhance the local blood circulation less than lidocaine. If

Table 1

Amount of lidocaine and prilocaine recovered from the tongues of rats.
A) 0.05 ml 1.0 solutions of ^{14}C-lidocaine or ^{14}C-prilocaine.
B) Injection of 0.05 ml of mixture of equal parts of 2.0 ^{3}H-lidocaine and ^{14}C prilocaine.
Each figure is the mean of values obtained from 8 animals.

Agent	Amount remaining in the tongue in % of injected dose		
	2 min.	4 min.	8 min.
A			
lidocaine	42.5 ± 1 1	20.7 ± 1 4	11.6 ± 0.9
prilocaine	47 6 ± 1 5	27 1 ± 1.5	16.3 ± 1.1
	0 05 > p > 0.01	0.01 > p > 0.001	0.01 > p > 0.001
B.			
lidocaine	50.3 ± 3 1	27.5 ± 3 1	10 9 ± 0.7
prilocaine	49.3 ± 3.4	26.4 ± 3 1	10.5 ± 0.6
	p > 0.05	p > 0.05	p > 0.05

a difference in action on the vessels caused this variation in rate of absorption, this difference would be expected to be lost if the agents were mixed and given in the same injection This possibility was studied in a separate group of animals When ^{14}C-labelled prilocaine and ^{3}H-labelled lidocaine were administered together the two agents could be analyzed simultaneously in the same extracts The results showed that in this experiment there was no difference in the rate at which the two agents disappeared from the injection site (table 1 B).

In a less richly vascularized area such as the sciatic nerve pocket in the rat, analysis of ^{14}C labelled prilocaine and lidocaine injected separately showed no statistically significant difference in the rate of disappearance from the injection site.

The *distribution* of ^{14}C prilocaine in various tissues was studied in rats at different times after administration by various routes. After intramuscular injection the concentration of prilocaine in the organs decreased in the following order lung, kidney spleen, brain, heart, liver and blood (table 2) The peak concentration in the lung was obtained earlier than in the other organs Only small amounts of the agent remained in the organs 120 min. after injection. The general pattern of distribution was the same as for lidocaine but higher concentrations of prilocaine than of lidocaine were found in the lung (difference highly significant) and brain (difference probably significant)

Table 2

Tissue distribution of prilocaine and lidocaine in rats at different times after intramuscular injection of 10 mg/kg.
Six animals were used for each determination.
The differences between the prilocaine and lidocaine concentrations were not significant unless indicated
(____ = $p < 0.001$ $0.05 > p > 0.01$).

Tissue	Amount recovered, µg/g fresh tissue									
	5 min		10 min		30 min		60 min		120 min	
	prilocaine	lidocaine	prilocaine	lidocaine	prilocaine	lidocaine	prilocaine	lidocaine	prilocaine	lidocaine
Blood	2.7 0.4	2.2 0.4	2.3 ± 0.2	2.4 ± 0.3	2.8 ± 0.1	3.6 ± 0.4	1.5 ± 0.1	1.5 ± 0.2	0.8 ± 0.1	0.6 ± 0.1
Brain	10.5 1.4	10.9 1.4	12.1 ± 1.0	10.8 ± 1.1	18.1 ± 1.1	13.6 ± 1.3	9.6 ± 0.9	6.6 ± 0.8	3.1 ± 0.3	2.9 ± 0.4
Liver	2.2 0.2	3.3 ± 0.3	2.7 ± 0.3	4.4 ± 0.4	7.3 ± 0.8	6.8 ± 0.9	2.2 ± 0.4	3.2 ± 0.5	0.8 ± 0.9	0.9 ± 0.1
Lung	31.1 ± 4.1	19.1 + 1.8	37.0 ± 2.6	20.7 ± 1.2	30.1 ± 1.7	16.8 ± 1.2	18.3 ± 1.6	[illegible] ± 1.3	5.2 ± 0.6	3.4 ± 0.5
Kidney	14.4 ± 0.9	15.8 ± 0.9	18.6 ± 1.3	21.1 ± 2.7	28.0 ± 2.7	22.7 ± 2.7	11.5 ± 1.1	11.5 ± 1.6	3.9 ± 0.4	4.1 ± 0.5
Heart	10.9 ± 1.0	10.5 ± 0.9	10.3 ± 0.6	11.9 ± 0.9	10.9 ± 1.0	10.7 ± 0.8	6.3 ± 0.6	4.5 ± 0.6	1.9 ± 0.3	3.1 ± 0.3
Spleen	8.6 ± 0.6	10.4 ± 0.7	13.7 ± 1.3	12.2 ± 1.0	19.3 ± 1.5	16.6 ± 2.4	10.7 ± 0.9	8.0 ± 1.1	3.9 ± 0.7	3.0 ± 0.4

In order to obtain the same rate of absorption for both agents from the injection site the two agents were injected simultaneously in a mixture of 5 mg ^{14}C-prilocaine and 5 mg ^{3}H lidocaine Determinations of the two agents could thus be done in tissue extracts from the same animals. The general pattern of distribution was the same for both agents and, as expected, the peak concentrations were observed earlier and were highest when the injections were given intraperitoneally (fig. 1). They were lower following intramuscular injection and lowest after subcutaneous injection.

The accumulation in the brain tended to be somewhat higher for prilocaine than for lidocaine (table 2) Therefore, the distribution of the drugs into this tissue was further analysed in another two series of experiments. In the first of these the contents of the agents in the brain of rats were determined when toxic symptoms (convulsions) had developed to the same degree. In order to produce comparable degrees of toxic symptoms, twice as much prilocaine as lidocaine had to be given. The results showed that at this time, the prilocaine concentration in the brain was more than twice that of lidocaine (97.6 ± 7.2 versus 45.5 ± 2.0 µg/g wet tissue, $p < 0.001$) Since double the dose was injected, the blood concentrations

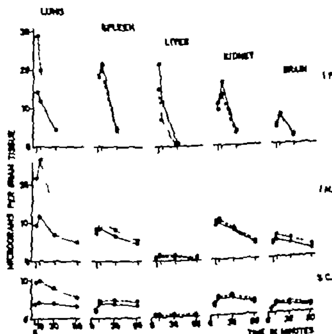


Fig. 1 Tissue distribution of lidocaine and prilocaine in rats after intraperitoneal, intramuscular and subcutaneous injection of a mixture of 5 mg/kg ^{14}C-prilocaine plus 5 mg/kg ^{3}H-lidocaine. O———O = prilocaine ●———● = lidocaine.

Table 3

Concentration of ^{14}C-prilocaine and ^{14}C-lidocaine in the brain of mice at the time of appearance and subsidence of convulsions.

Agent	Dose mg/kg i.p.	Appearance of convulsions: onset time min.	Amount recovered µg/g	n	End of convulsions: duration min.	Amount recovered µg/g	n
Prilocaine	200	4.8 ± 0.4	115.6 ± 14.6	16	13.6 ± 0.6	92.0 ± 9.2	8
Lidocaine	100	3.3 ± 0.3	43.1 ± 2.8	12	18.2 ± 2.0	29.1 ± 1.7	8

of prilocaine were also about twice as high in these animals (27.6 ± 3.1 versus 14.1 ± 1.3 µg/g). In the second series, mice were used and the concentration of prilocaine and lidocaine in the brains determined at the time when convulsions appeared as well as at the time when they ceased. As shown in table 3 the prilocaine concentration in the brain was about three times higher than that of lidocaine both when the convulsions appeared and when the toxic symptoms subsided. The convulsions following prilocaine however lasted about 5 min. less, starting later and ending earlier than those following lidocaine. In this particular series the toluene extracts of lidocaine had been washed three times in phosphate buffer pH 6.2 (SUNG & TRUANT 1954) to exclude any possible amine metabolites of lidocaine (HOLLUNGER 1960a).

Local distribution between a nerve and its surrounding tissue was studied in rats injected at mid thigh level in order to produce sciatic nerve block. When the motor paralysis subsided, there was more prilocaine than lidocaine left in the nerve (table 4). The figures for the amounts left in the surrounding muscle tissue were variable and no statistical difference was observed. It should be noted, however, that the muscle concentrations were obtained about 70 min. after the injection of prilocaine and after about 56 min. in the case of lidocaine.

Elimination in urine was studied in a series of 10 rats with ^{14}C-labelled prilocaine injected intraperitoneally. About 25 % of the injected radioactivity was recovered from the urine collected in ethanol up to 6 hours after the injection. After alkalinization and extraction only about 2 % of the injected radioactivity was recovered, the main part of this being in the samples taken within 2 hours after the injection. The major part of the excretion in urine thus consisted of metabolites of prilocaine. The faeces

Table 4

Amount of ^{14}C-lidocaine nd ^{14}C-prilocaine in the sciatic nerve and the surrounding muscles at the end of sciatic nerve block in rats produced by 0.2 ml of a 1.0% solution.

Compound	µg/g tissue		Mean duration of block in min.	Number of animals
	nerve	muscle		
Lidocaine	74 4 ± 7 5	205 4 ± 52.5	55.8 ± 1.4	14
Prilocaine	107 6 ± 8.7	177 7 ± 49 6	70._ ± 4.3	15
	0.01 > p > 0.001	p > 0.05	p < 0 001	

contained no detectable amounts of unaltered prilocaine or radioactive metabolites.

Metabolism The rate of metabolic transformation of prilocaine *in vivo* was studied in whole animals (mice) at different times after intraperitoneal injection. The amount of ^{14}C prilocaine that could be recovered rapidly decreased with time (fig. 2) Since the elimination by the urine and faeces

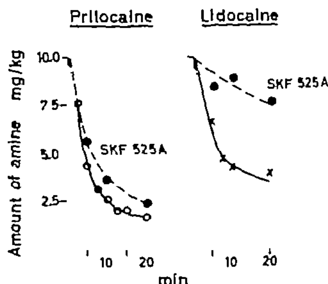


Fig. 2. Rate of metabolism of prilocaine (○) and lidocaine (×) in mice *in vivo*. Amount of prilocaine and lidocaine recovered from whole animal at different times after injection of 10 mg/kg i. p. nd corresponding values after pretreatment with *SKF 525A* (25 mg/kg) injected one hour before the local anesthetics. Each point is the mean value from 5 animals.

Table 5

Per cent ^{14}C-prilocaine and ^{14}C-lidocaine metabolized by liver homogenates and slices from different animal species. 48 µg of the agents were incubated at 37° for 10, 20 or 30 minutes.

Species	Homogenates				Slices			
	prilocaine		lidocaine		prilocaine		lidocaine	
	10′	30′	10′	30′	10′	20′	10′	20
Mice	25	45	4	5	59	74	10	22
Rats	17	31	5	10	–			
Guinea-pigs	46	58	3	4	–		–	
Rabbits	63	74	6	11	70	85	13	35
Cats	13	28	5	7	21	55	17	33

of the unaltered compound was quite small this finding indicated a rapid rate of metabolism The rate of the *in vivo* decomposition of ^{14}C prilocaine was practically unaffected by the enzyme inhibitor SKF 525A (AXELROD, REICHENTHAL & BRODIE 1954) which, on the other hand greatly retarded the metabolism of lidocaine in a similar study

In vitro studies were performed with both homogenates and slices. ^{14}C prilocaine was quite effectively transformed by liver homogenates and slices from different animals while ^{14}C-lidocaine incubated in an identical manner was less affected (table 5) Pretreatment of mice with SKF 525A, 25 mg/kg intraperitoneally one hour before preparing the liver homogenate, did not significantly reduce the capacity of the homogenate to metabolize prilocaine (45 % metabolized in 30 min as compared to 53 % in the controls)

Lung and kidney homogenates and slices from rabbits and cats were also capable of metabolizing prilocaine to a considerable degree (table 6) Some transformation of lidocaine also occurred with slices from the lungs and kidneys from cats. No detectable activity was found in homogenates of brain heart, skeletal muscle or blood serum.

The radioactive metabolite formed by rat liver slices from ^{14}C prilocaine was insoluble in ethyl ether (pH 10) but was water soluble and on paper chromatography using different solvents (I–III cf methods) the Rf values for the metabolite were similar to those of N propylalanine. When tritiated compounds were used one of the radioactive breakdown products of prilocaine formed by liver slices could be extracted by ethyl ether at pH 10 which on paper chromatography (solvents I and III) had Rf-values similar to those of o-toluidine These results indicated that

Table 6

Per cent ^{14}C-prilocaine and ^{14}C-lidocaine metabolized by kidney and lung homogenates and slices from rabbit and cat. 48 μg of the agents were incubated at 37° for 10, 20 or 30 minutes.

Species	Homogenates				Slices			
	prilocaine		lidocaine		prilocaine		lidocaine	
	10′	30′	10	30′	10′	20′	10′	20′
Kidney								
rabbits	40	54	0	0	41	59	0	0
cats	27	54	8	18	49	63	34	42
Lung								
rabbits	27	48	0	0	38	65	0	2
cats	18	38	5	10	57	71	15	31

prilocaine was metabolized by hydrolysis of the amide linkage. Further studies of the metabolites of prilocaine are, at present being made

The formation by liver slices of aromatic, radioactive metabolites from ^{3}H-prilocaine was more rapid than from ^{3}H-lidocaine (fig. 3) A decrease

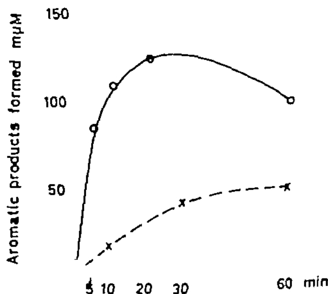


Fig. 3 *In vitro* metabolism of ^{3}H-prilocaine and ^{3}H-lidocaine by mouse liver slices. 250 mg slices incubated with 200 mμmol of the agents at 37°. The aromatic products formed are estimated as o-toluidine (O) and 2,6-xylidine (×) respectively

in the formation of the o-toluidine like product was noted with longer times of incubation which may indicate a further metabolic transformation.

Discussion

The absorption studies have shown that in a richly vascularized tissue such as the tongue of rats, it is possible to demonstrate that lidocaine is more rapidly absorbed than prilocaine. This difference was eliminated when the two agents were administered simultaneously in a mixture. Thus the agents seem to influence the small vessels and thus the tissue clearance to different degrees. It has previously been reported that lidocaine seems to enhance the local circulation more than prilocaine (ÅSTRÖM & PERSSON 1961).

The conclusion that lidocaine and prilocaine differ in their action on small blood vessels is supported by some unpublished studies in which the reactions of the blood vessels of rat mesoappendix were directly observed according to ZWEIFACH (1948). Prilocaine in a 2% solution was usually without effect while lidocaine invariably produced vasodilatation.

It should be emphasized that with the technique used, a slower rate of absorption of prilocaine from an injection site could not be directly demonstrated in a less richly vascularized region such as the muscles of the sciatic nerve pocket in rats. Yet prilocaine remained at a higher concentration than lidocaine for a longer period in the thigh portion of the sciatic nerve. This finding could thus not be explained by a sustained higher concentration of this agent in the tissues surrounding the nerve. It would seem rather to indicate that the prilocaine molecules by some mechanism not as yet established are distributed to a greater extent to the sites of action in this type or nerve. The difference in distribution does not seem to be in a simple manner related to lipid solubility since the distribution coefficient cod liver oil/water (BRÄNDSTRÖM 1963) is higher for lidocaine (F = 36) than for prilocaine (F = 23) (BRÄNDSTRÖM, personal communication). The observation that at the time when the sciatic nerve block in rats subsided the concentration of prilocaine in the nerve was higher than that of lidocaine indicates that in order to produce the same degree of block, the concentration of prilocaine in the nerve has to be higher than that of lidocaine. The relationship between "potencies" of these agents on this mammalian nerve therefore seems to be the same as that found in the isolated frog nerve (ÅSTRÖM & PERSSON 1961). These findings would also seem to be of relevant for the clinical observations e g by ERIKSSON & GORDH (1959) that prilocaine without a vasoconstrictor produces finger blocks of a longer duration than lidocaine in plain solutions.

The acute toxicity of local anaesthetic agents seems to depend on a direct action on the central nervous system (GOODMAN & GILMAN 1965) The concentration of the agents present in the brain would be expected to vary with the route of administration and also with the affinity of the agents for the brain and other organs. The analysis of the distribution pattern for prilocaine and lidocaine showed no major differences between the two agents with the exception of the lung which regularly showed higher concentrations of prilocaine than of lidocaine and of the brain, when the difference was in the range of significance. These findings like the sciatic nerve block experiments suggest that the two agents may differ with regard to their affinity for certain tissues.

LUDUENA, HOPPE & BORLAND (1958) have suggested that the acute intravenous toxicities of local anaesthetic agents is closely correlated to their local anaesthetic effect. In the case of prilocaine and lidocaine the i. v toxicities are poorly correlated to their local anaesthetic effects as measured by most *in vivo* tests. Despite almost equal effectiveness of the two agents (ÅSTRÖM & PERSSON 1961 ERIKSSON & GORDH 1959 CRAWFORD 1964) prilocaine is about half as toxic as lidocaine (WIEDLING 1960 ÅSTRÖM & PERSSON 1961 ENGLESSON *et al* 1962) The studies reported here have shown that prilocaine has to reach a concentration at least twice that of lidocaine in the brain, to produce a similar degree of toxic symptoms. The toxicity of these agents seems to be better correlated to their potency on the isolated nerve where prilocaine is about 0 6 as active as lidocaine (ÅSTRÖM & PERSSON 1961). It would thus seem that there is a correlation between acute i. v toxicity and local anaesthetic effect, if the latter is determined on a preparation such as the isolated frog nerve, *i e* where effects on local blood circulation, diffusion through tissue membranes and other factors do not modify the situation. This conclusion would seem to explain why in the study by LUDUENA, HOPPE & BORLAND (1958) the correlation between the toxicity and anaesthetic effect was particularly good ($r = 0.97$) if the effect was determined intraspinally in rabbits but less marked ($r = 0.74$) if it was measured intradermally in guinea pigs.

The metabolic studies have indicated that the secondary amine prilocaine is rapidly metabolized and enzymatically split at the amide bond by an amidase present mainly in the liver This finding has previously been reported in a preliminary form by ÅSTRÖM (1965) and independently by GEDDES (1965). An amidase similar to that in the liver and capable of hydrolyzing prilocaine has previously been demonstrated in kidney slices by GEDDES (1965) In the present study this finding has been confirmed and in addition it has been shown that a similar activity is also present in lung tissue.

In the case of the tertiary amine lidocaine, it has previously been shown that an oxidative de-ethylation with the formation of monoethylglycine xylidide precedes the splitting of the amide linkage (HOLLUNGER 1960a). Our observation that SKF 525A in rats and mice significantly retarded the metabolism of lidocaine both *in vivo* and *in vitro* is of particular interest. SKF 525A, known to be an inhibitor of oxidative liver enzymes bound to the microsomes, has previously been found to retard the rate of hydrolysis of monoethylglycinexylidide at the amide linkage *in vitro* (HOLLUNGER 1960b). The results in this investigation showing that SKF 525A *in vivo* significantly reduced the rate of metabolism of lidocaine but not that of prilocaine, would seem to indicate that at the concentrations used, the enzyme responsible for the oxidative de-ethylation of lidocaine was inhibited, while the amidase was largely unaffected and still capable of hydrolyzing the secondary amine prilocaine at an apparently unchanged rate. It should be added that any monoethylglycinexylidide formed in all experiments reported here was recorded as unchanged lidocaine due to the extraction procedures used, unless otherwise stated.

The finding that prilocaine is broken down in the organism more rapidly than lidocaine would seem to be one factor of importance for the toxicity of these agents. Another important finding was that prilocaine had a lower toxic potency *per se* on the central nervous system as evidenced by the distribution in the brain. *This factor seems to be especially important* when the agent enters the systemic circulation rapidly, for instance with an accidental intravenous injection.

Summary

Some factors determining the action and toxicity of local anaesthetics have been studied with the two almost equally effective agents, prilocaine and lidocaine.

Prilocaine was less rapidly absorbed than lidocaine from the tongues of rats, but not significantly so from less vascularized regions like the sciatic nerve pocket. The concentration of prilocaine in the sciatic nerve of rats at the time of recovery from motor block was, however, higher than that of lidocaine. These studies indicate a difference in distribution to the site of action and also that the relationship between the concentrations of these agents required to produce a block *in vivo* is similar to that previously found *in vitro*.

Following injection by various routes in rats, the two agents are distributed to various tissues in a similar manner with the exception of the lungs where the concentration of prilocaine was regularly higher than that of lidocaine. The concentration of prilocaine in the brain also tended

to be somewhat higher that that of lidocaine. After the injection of equally toxic doses the concentration of prilocaine in the brain was more than twice as high as that of lidocaine at the time of appearance as well as of disappearance of convulsions. The convulsions after prilocaine started later and ended earlier than after lidocaine.

Only small amounts of intact prilocaine were excreted in the urine and no detectable amount in the faeces. Prilocaine seems to be a better substrate than lidocaine for the amidase found mainly in the liver but also present in kidney and lung tissue. Contrary to lidocaine, the break down of prilocaine was largely unaffected *in vivo* as well as *in vitro* by SKF 525A. Separation by paper chromatography has indicated that two of the metabolites of prilocaine have the same Rf values as o-toluidine and N propylalanine.

The fact that prilocaine is less than half as toxic as lidocaine seems to be due to a weaker action on the central nervous system and to a rapid metabolic transformation to products with low acute toxicity The studies indicate that the toxicity of local anaesthetic agents does not have to be correlated to their *in vivo* effectiveness.

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From the Department of Pharmacology University of Uppsala, Sweden
(Head Professor Ernst Bárány)

Estimation of Brain Sensitivity to Hexobarbitone (Enhexymal NFN) in Rats by an EEG Threshold

By

Göran Wahlström

(Received April 18 1966)

The effects of barbiturates are often evaluated experimentally as the sleeping time Sleeping time in this connection usually means the time during which the righting reflex is absent and is consequently a measure of the time under anaesthesia. This is delineated by two events, a) the loss of the righting reflex and, b) the return of this reflex. The induction time i.e from the time of injection to the loss of the righting reflex has an effect on the measurement. It is often of such short duration (depending on the barbiturate) when compared with the sleeping time that changes in induction time are usually neglected. Although values of the induction time are seldom given in the literature, they can sometimes definitely influence the sleeping time (WAHLSTRÖM 1966)

Sleeping time measurements are thus usually determined by the end point, i e. the return of the righting reflex. As this end point depends not only on the central nervous system sensitivity to the barbiturates but also on the rate of elimination, sleeping time measurements are often inadequate for studying the central nervous system sensitivity to barbiturates more exclusively This is especially clear in connection with barbiturate tolerance development and where barbiturates are combined with other drugs. Both of these phenomena influence the rate of elimination by increasing or decreasing the metabolism of the barbiturate and thus affect the sleeping time (REMMER 1962 CONNEY *et al* 1960 LAROCHE & BRODIE 1960).

Compared with the number of studies in which sleeping time has been used there are few studies in which an attempt has been made to evaluate the induction of the anaesthesia more exclusively (SHAGASS *et al* 1962 MCCANCE 1964). If the central nervous system sensitivity is the main

object, then the measurements of the induction of anaesthesia have the advantage that the rate of elimination does not *appreciably influence* them.

In the present paper an anaesthesia threshold method is presented. The changes *during the induction of hexobarbitone (enhexymal NFN)* anaesthesia by a continuous infusion have been followed with the electroencephalogram (EEG) and this was used to determine the threshold. It was *developed in order to follow changes in the sensitivity of the* central nervous system to barbiturates over a long period in intact ani mals.

Methods

Male Sprague-Dawley rats were used. The initial weight of the animals before the first threshold determination was around 300 g. The rats were kept in oom t constant temperature f 30° The light in the room (two fluorescent lamps) was regulated with 12 hours of light and 12 hours of darkness in each cycle Th light was turned o at 8 a.m. The rats had access to water and food pellets d libitum.

The hexobarbitone sodium (evipan ®) was btained from Bayer AG as a powder All doses were calculated as the sodium salt. The barbiturate was diluted t concentration of 100 mg/ml with distilled water before use. This solution was never kept for more than 1 hour From this solution an amount was taken to give each ndividual rat dose of 150 mg/kg. This solution was then further diluted with 0.25 % sodium chloride t give a final volume of 1 0 ml.

The infusion was given int *tail vein by moto -driven injection pparatus* which has been described by Öbrink (1948). The standard speed was set to 0.1 ml/min with which the solution of hexobarbitone used corresponded t 15 mg/kg/min (0.25 mg/kg/sec). After the infusion the tail veins were inspected to see f white spot had ppeared hich is a sign of subcutaneous jection. I doubtful cases suction for blood performed after the threshold had been determined This was alw ys done if more tha 0.5 ml (75 mg/kg) had been infused. I these cases care as always taken to ensure that no leak had *occurred. After each* threshold determina on all the tails ere examined for necrosis. This was usually done the next time the rat was used Threshold determinations which ere known to have been subcutaneous alway produced necrosis. Thus determinations i which necrosis was subsequently observed were always excluded.

A Schwarzer electroencephalograph *was used for the EEG recordi g. Paper-speed* as 3 cm/sec. I the preliminary trials and the first experiments come stainless steel screws were used as electrodes. These were screwed into holes drilled in the skull of the rat, and further fixed by phosphate cement. Care as taken not t penetrate the dura. The operation was performed t least 1 week before the first threshold determination. After all experiments ere finished the inside of the skulls ere exam ned particularly for infection, hemorrhage or perforation of the dura.

The *stainless* steel electrodes ere not suitable for long term experiments (over several months), as they often became loose and could not be replaced ithout interrupting the experiment. The threshold chosen (silent second, see result part A) detectable ith subcutaneous metal sutures as electrodes, and these could easily be replaced. Silver wire as first used but was *too fragile* and t *present* stainless steel ire is being used I The rats were sutured immediately before the first threshold determination and the

were subsequently replaced when needed. With this kind of electrode few curves were impossible to read but the loss was small when compared with the gain in simplicity. The animals were earthed separately from the nose and usually only one bipolar recording was taken from electrodes one on each side of the mid line.

The rats were restricted in a wooden box during the threshold determination. The box was open at the head and tail end. The head part consisted of a cloth nailed to the box and tied around the neck of the rat. Care was always taken not to restrict respiration, and free respiration was also present during the threshold determination.

The threshold was usually determined in two rats at the same time. After the chosen end point in the electroencephalogram (EEG) had appeared, the infusion was discontinued and the time without righting reflex (sleeping time) was measured. The results of this part of the experiment will be published separately (WAHLSTRÖM 1966).

Results

A *Description of the changes in the electroencephalogram*

During a continuous infusion of hexobarbitone with the chosen speed of 15 mg/kg/min. there are some changes in the EEG which normally appear after a fairly constant dose has been infused. These EEG changes are used as an indication of the effects on the central nervous system. The changes are illustrated in Fig. 1. The pre-experimental EEG (A) has a rather low amplitude and a frequency around 30 cyc./sec. This agrees well with reported EEGs in the alert rat (BRADLEY *et al.* 1960, FUKUDA *et al.* 1959). After approximately one minute (15 mg/kg hexobarbitone) there is an increase in amplitude and at the same time a decrease in frequency (B and B_1). The change is usually gradual and takes 10–20 sec. before it is established. This part of the record is often disturbed by jerks

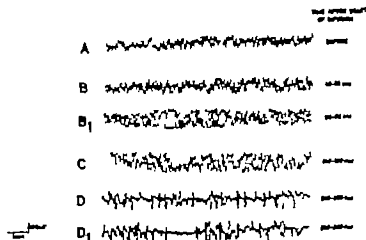


Fig. 1. The electroencephalogram of a male rat during a continuous intravenous infusion of hexobarbitone (0.25 mg/kg/sec). Electrodes: steel screws.

and sometimes short convulsions. These usually have disappeared when 30 mg/kg of hexobarbitone has been infused.

At this stage the frequency of the high potentials has decreased (C) and after 45 mg/kg there is a tendency for them to appear in bursts with increasing periods of burst suppression (D). The rats have at this stage lost the righting reflex. Usually when about 60 mg/kg of hexobarbitone has been infused, a burst suppression of 1 sec. or more appears in the record (D_1) and if the infusion is continued the periods of burst suppression increase still more.

B *Definition of the chosen threshold, the silent second*

Visual inspection of the EEG recordings during a continuous infusion of hexobarbitone thus reveal two kinds of changes which are distinct enough to make it possible to use either of them as the end point in a threshold study These changes are 1) the conversion of low amplitude fast activity to slower activity with higher amplitude and 2) some pre determined duration of burst suppression

The conversion from low amplitude fast activity to slower activity with higher amplitude has several drawbacks as a threshold. It is often masked by artefacts due to movements. The change is not clear cut but gradual and can thus be influenced by subjective evaluation. Due to the small amount of hexobarbitone infused, this subjective evaluation can lead to a substantial error as a 10 sec. difference in the estimate of the change corresponds to approximately a 20 / difference in the threshold dose. The low threshold dose also gives a high relative importance to small errors which are independent of the amount infused, such as the estimate of the injected volume or changes in circulation time. Another reason why threshold based on the change in amplitude is not used, is that it can not be combined with measurements of the sleeping time since the animals have not lost their righting reflex at this stage.

A pre-determined duration of burst suppression does not have these drawbacks It is easy to evaluate objectively and occurs when more hexobarbitone has been infused. When burst suppression appears the animals have lost their righting reflex In preliminary trials it was found that the earliest most distinct burst suppression seen had a duration of more than 1 sec. and the threshold used was consequently defined as the amount of hexobarbitone needed to obtain a burst suppression with a duration of one second or more. *This threshold burst suppression has been denoted as the "silent second"*

The development of burst suppression up to the silent second is shown in Fig. 2. The figure is based on the second EEG recorded from 51

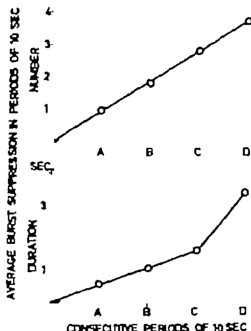


Fig. 2. Number and duration of burst suppressions in successive 10 sec. periods during continuous i.v. infusion of hexobarbitone. The time of the infusion was divided in 10 sec. periods from the start. Period D is the one in which the silent second occurred. Period C is the one immediately preceding D and so on. Burst suppressions smaller than 0.4 sec. have not been included. Before period A there were only two short burst suppressions in all the records studied. D is obtained from the second EEG performed in 31 rats.

It is seen that there is a steady linear increase in the average number of burst suppression from A to D. The average duration of burst suppression shows a similar linear increase from A to C which is slightly more than 0.5 sec./ten second period. Between C and D there is however a sharp increase in average duration.

The reason for this difference between average number and average duration of the burst suppressions is shown in Fig. 3 where the frequency distribution of the duration of each burst suppression has been plotted for periods A, B, C, and D. In period A, B and C the shape of the distribution was similar. In period D the pattern of the distribution was however changed to one with a clear tendency towards a second rise around a duration of 1.3 sec. This indicates that the silent second can be more than just an arbitrary threshold, in a rising population of burst suppressions with increasing duration. Two possible explanations are 1) that there are two distinct populations of burst suppression one with a short duration and one with a long duration, and 2) that to give the silent second there is a sudden melting together of two or more shorter periods of burst suppression.

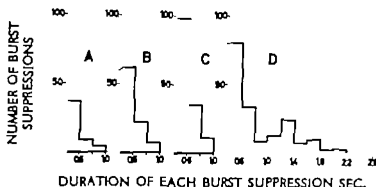


Fig. 3. Frequency distribution of burst suppression duration in successive periods of 10 sec. The letters indicate the 10 sec. periods as in Fig. 2 The burst suppressions have been obtained from the second EEG performed 51 rats. Burst suppressions smaller than 0.4 sec. have not been included.

C. *Properties of the threshold using the silent second*

The distribution of the amount of hexobarbitone needed to obtain the silent second is shown in Fig. 4 Four threshold determinations were performed after each other in all rats. The rats had never been used in any experiment before the first determination. The material consists of rats with satisfactory records during the first four determinations, which later participated in various experiments during the last three years.

As can be seen in Fig. 4 the distribution of the dose needed to obtain a silent second in determination B II B III and B IV were essentially the same. The means were also of approximately the same magnitude. The distribution and mean obtained in determination B I were however different from the others. On the average, more hexobarbitone was needed to obtain the silent second. The distribution has a larger range and standard deviation but there was no shift of the lower extreme as compared with determination B II B III and B IV This difference between the first and the following determinations can be due to excitement caused by suturing before the first determination, in which all rats participated and the unfamiliarity with the test situation. This conclusion is also substantiated by observations on the behaviour of the rats. The rats were definitely more excited before the first determination than before the following determinations.

Suturing alone does not explain the differences, as the threshold in a group of 24 rats re-sutured for the first time was 105.3 ± 3.1 (s.e.m.) % of a previous threshold before which no suturing had been needed This first re-suturing (silver wire) was performed approximately 3 weeks after the initial sutures were placed

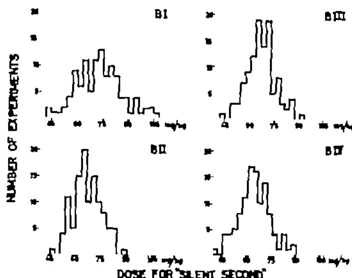


Fig. 4. Frequency distributions of the doses needed to obtain the silent second. Four successive determinations in 110 rats have been used. Determination B I is the first one ever performed in these rats. The average time between determination B I and B II was 14 days, between B II nd B III 10 days and between B III and B IV 9 days. The mean and standard deviation determination B I 73.1 ± 13.1 mg/kg, B II 66.8 ± 8.1 mg/kg, B III 66.6 ± 8 7 mg/kg and B IV 64.4 ± 9.3 mg/kg.

The amounts of hexobarbitone needed to obtain the silent second in determinations B II–B IV have been further analysed in Fig. 5. The

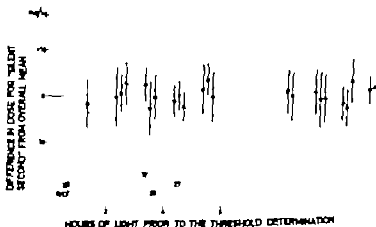


Fig. 5 The influence of hours of light *prior* to the threshold determination on the dose needed for the silent second. Determinations B II, B III and B IV (Fig. 4) have been used. Differences between the verage threshold doses obtained in each hour after the light was turned on and the overall mean for the corresponding determination have been calculated. The number f rats for each point and the symbols used are shown in the lower part of the figure. Twice the standard error is indicated by the bars.

records have been divided into groups according to how long the light had been on in the room before the threshold determination was performed. It is clearly seen from Fig. 5 that the period of light before the determination had no effect on the results, as all values are distributed around the overall mean with no tendency for a maximum or minimum. During the light period of 12 hours (inactivity of the rats) there was thus no change in sensitivity to hexobarbitone in the central nervous system with the silent second as the criterion

Experiments B II–B IV have been used to see whether the calculation of the threshold dose as a function of body weight was correct. The distributions of the threshold doses are seen in Fig. 4 The means and ranges of the body weights in B II were 333 (255–415), in B III 348 (260–435), and in B IV 361 (275–445) g. The correlation coefficients between weight and threshold dose were in B II 0 02, in B III −0 02 and in B IV 0 09 Thus there was no residual influence of body weight on the threshold dose.

As the same rats participated in all threshold determinations in Fig. 4 the correlation coefficients between the thresholds have been calculated for determinations B II, B III and B IV The figures obtained are between determinations

	B II–B III	B III–B IV	B II–B IV
r =	0.33	0.27	0.25
P =	<0 001	<0.01	<0.01

The individual rats thus tend to keep to a certain threshold level. This is further strengthened by the evidence presented in Table 1

In Table I another series of determinations is shown which was not included in Fig. 4 In this series 9 threshold determinations were performed in 7 rats. The first determination was discarded and the others followed at intervals of 2–3 days. The means of the eight determinations evaluated had a range of 57 0–79 7 mg/kg for the individual rats. The

Table 1

Analyses of variance on 8 threshold determinations in 7 rats.

Source of variation	DF	Mean square	F	P
Rats	6	482.69	18 97	<0.01
Days	7	23.33	0.92	>0 05
Residual	4	25 45		

Table 2

Long term changes in threshold dose.

Experiment	Pre-experimental average mg/kg	Number of rats	Time without experiments Months	Threshold dose as per cent of pre-experimental average Mean	s.e.m.
I	65.0	12	3.3	99.3	2.5
II	68.7	12	4.0	103.6	3.0
III	61 7	8	4 7	101.9	7.3
			Overall mean	101 6	

range of the means for the experimental days was 63.8–68 7 mg/kg. An analysis of variance was performed and the result are shown in Table 1 This analysis shows that there is no significant variance between days but a significant one between rats The individual rats thus tend to have a threshold dose of hexobarbitone for the silent second which is fairly constant over a short period of time (2–3 weeks) but the threshold dose can vary considerably between the rats.

Three experiments have also been performed to evaluate the long term changes in the threshold dose. In all the rats used the first threshold determination was discarded. Further threshold determinations were performed until each rat had three determinations which were acceptable (in a few cases there were only two) The average dose obtained from these determinations was used as the pre-experimental average After the last determination used in the pre-experimental average the rats were left undisturbed for 3–5 months. A new threshold determination was then performed and the dose obtained was calculated as the per cent of the pre-experimental average The results are shown in Table 2. It is quite clear that 3–5 months without any treatment had no effect on the threshold dose. The threshold dose is thus fairly constant over several months in adult male rats.

To see whether cerebral blood flow is a limiting factor in the present experiments, the effect of 5 / carbon dioxide in the inspired air was tested in seven rats. This amount of carbon dioxide gives an increase in cerebral circulation (SOKOLOFF 1959) The carbon dioxide-air mixture was given to the rats for four minutes before and during the threshold determinations. Compared with one normal threshold dose obtained 3–5 days before the carbon dioxide experiment 94 1 ± 5.2 / of hexobarbitone was needed in the carbon dioxide experiments. A slight re-

duction was thus obtained but with a large standard error Thus the change in cerebral circulation induced by 5 / carbon dioxide did *not* appreciably influence the threshold

D *Effect of various infusion rates on the threshold dose*

A series of experiments have been performed to determine the effect of the infusion rate on the dose needed to obtain the *silent second* In these experiments one threshold determination with the usual infusion rate, 0.25 mg/kg/sec. (*standard rate*), was first performed. After three to five days a new determination was performed with the infusion rate to be tested and the results were calculated as the per cent of the standard rate dose. The results are shown in Fig. 6. It is evident from Fig. 6 that there is an optimal infusion rate which is close to the one chosen for the normal threshold determinations (0 25 mg/kg/sec.). With slower infusion rates below 0 10 mg/kg/sec. there is a marked increase in the threshold and with faster infusion rates there is a slower and approximately linear increase in the threshold.

With a slow infusion rate there is thus a slower accumulation of hexo-

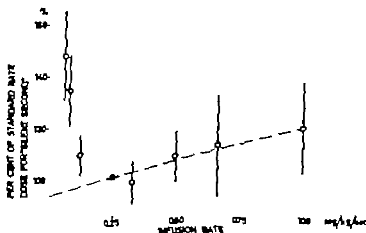


Fig. 6. The effects of different speed of infusion on the dose needed to obtain the silent second. The concentration of hexobarbitone was kept constant. Filled circle denotes standard rate (0.25 mg/kg/sec). The standard rate dose for silent second and umber of rats from which the percentage has been calculated is shown below. Twice the standard error is indicated by the bars.

Rate	1.0	0 67	0.5	0.33	0.125	0.08	0 06	mg/kg/sec
Dose	62 7	61 4	59.0	59 7	64.2	63 0	62.9	mg/kg
n	11	13	11	16	13	14	14	

barbitone in the central nervous system than expected. Thus slower accumulation must partly be due to an increase in the distribution of hexobarbitone to other parts of the body during a slow infusion. It is also obvious that with the slowest infusion rate 0.06 mg/kg/sec. the catabolism of hexobarbitone can influence the increased amount needed, since the total time of infusion before the silent second appeared was approximately twentyfive minutes.

Some of the increases seen with faster infusion rates must be due to the increased amount of hexobarbitone in the vascular system which had not yet reached the central nervous system at the time of the silent second. This amount corresponds to the amount infused during one circulation time (tail to brain) in the rat. If the only reason for the increase was this delay then the excess dose needed above that at the standard rate would be circulation time times excess rate. Using 0.25 mg/kg/sec. as the basal rate this calculation for the three highest rates gives the following values of the circulation time 12, 14 and 19 sec.

Separate experiments were performed to evaluate the circulation time in the rat by rapid injection of 0.15 ml of 40 % fluorescine sodium (W/V) in the tail vein and observing the time before the fluorescense appeared in the nose (ultraviolet illumination). The experiments gave an average circulation time tail to nose of 13.3 (n = 12) sec. with a range of 9–25 sec. The figures obtained are of the same magnitude as those obtained by the indirect calculations using the increased threshold. Thus the threshold increase seen with faster infusion rates can be fully explained by the increased amount which had not reached the central nervous system, when the silent second appeared.

The approximately linear increase in the dose of hexobarbitone needed to obtain the silent second can then be used for estimating how much the recorded threshold dose exceeds the true one, by using the interception with the y axis (Fig. 6 dotted line). At this point the excess dose with 0.25 mg/kg/sec. due to the delay in the circulation is corrected. As can be seen from Fig. 6 this excess is 5–10 %.

In the experiments shown in Fig. 6 the rate of the hexobarbitone infusion was changed by changes in volume rate, the concentration being kept constant. Two experiments were performed in which the hexobarbitone infusion rate was changed by means of alteration in concentration. The rates used were 0.125 mg/kg/sec. and 0.333 mg/kg/sec. The slow rate was achieved by using hexobarbitone in such a way that the amount in 1 ml corresponded to a dose of 75 mg/kg and in the fast rate experiments in such a way that the amount in 1 ml corresponded to a dose of 200 mg/kg. The additional dilution needed in the slow rate experiments was done with 0.9 % sodium chloride.

The results have been calculated as the per cent of a previously performed threshold determination with the standard rate in the same rat. The mean and the standard error in the slow rate experiments were 95.3 ± 7.5 (n = 10) and in the fast rate experiments 105.0 ± 4.2 (n = 11). Compared with the corresponding results obtained with the constant concentration (Fig. 6) none of the differences were significant (student's t-test). Change in hexobarbitone infusion rate by means of a change in concentration or by a change in volume rate thus tend to give similar results.

Discussion

The electroencephalogram (EEG) has two advantages in a threshold method for determining central nervous system sensitivity to a depressive agent. One is that it makes a continuous recording possible without disturbing the animal. If the depression is estimated by some other method, as for instance the loss of a reflex, this must be tested and the animals consequently disturbed. The loss of sensitivity in the test must be continuously balanced against the risk of influencing the test by stimulating the animal. One example is given by McCance (1964) who used a continuous infusion of hexobarbitone in mice with loss of righting reflex as the threshold. The test could not be performed continuously and thus a certain variable excess in the dose was inevitable as "It was considered important not to overstimulate the mouse."

The other advantage with the EEG is that it directly records the activity of the brain which is the organ of interest. It is true that if the primary effect of the barbiturate were on one well localized part of the brain the EEG might be too crude a measure of the effect. Killam (1962) in a review of drug action on the brain stem reticular formation states that at least part of the sedative and anaesthetic effect of barbiturates is obtained by effects on the reticular formation. However, the depressant effects on the central nervous system is by no means restricted to this system (Domino 1962).

The likelihood of the EEG being too crude is reduced by the use of the silent second in which a burst suppression is the threshold. The exact cause of the phenomenon of bursts at this stage of anaesthesia is not known (Faulconer & Bickford 1960). The bursts are however well synchronized at different levels of the human brain during deep barbiturate anaesthesia (Bickford *et al.* 1953). Such a synchronization is also seen during barbiturate anaesthesia in the dog (Swank 1949). As there is no reason to assume that the rat behaves differently, the silent second very probably measures a generalized change in brain activity. It

possible that more subtile threshold methods could be developed with deep electrodes, and that with such threshold methods it would be possible to establish the relative sensitivity to various drugs in different parts of the brain. But surface EEG leaves the brain intact and at present it seems to be the best recording method for general pharmacological purposes.

For a barbiturate to be useful in a continuous infusion as a test of central nervous sensitivity it must be able to penetrate the brain rapidly Hexobarbitone is classed as such a substance (BUSH 1963) and the fact that a threshold only slightly in excess of the theoretical one (overshoot less than 10 %, Fig. 6) was obtained with hexobarbitone in the present experiments shows that "penetration time" plays a minor role as compared to circulation time.

The EEG was previously used by SHAGASS *et al* (1962) to obtain a thiopentone threshold in the rabbit. The method was adapted from a similar one used in the human (SHAGASS 1954) The end points used were maximum and fastest increase in integrated EEG activity between 17 and 32 cyc./sec. Head drop was also tested at an interval of 30 secs. The injection was discontinuous and the amounts injected were 1 or 2 mg/kg every 30 sec. This method of injection limits the threshold to steps which correspond to approximately 10 or 20 / of the recorded thresholds. If the EEG activity has to be integrated to obtain a threshold it may be hard to avoid a stepwise injection to bypass the time element in the integration. But the brain concentrations after each step can by no means be assumed to be stable with a barbiturate like thiopentone which rapidly penetrates and leaves the brain It is possible that the disappointingly low overall reliability found by SHAGASS *et al* between the tests and between the same tests in two succeeding experiments may partly be due to the stepwise injection. Since this tends to give fluctuating levels of thiopentone, a threshold based on a critical brain concentration will be obscured. The higher concentration which exaggerates the steps also gave more inconsistent results.

With the present method there is an optimal injection rate around 0.25 mg/kg/sec. of hexobarbitone (Fig 6) Similar experiments were done by MCCANCE (1964) with the method described above. MCCANCE used a factorial arrangement with four replications which included infusion rate and concentration of hexobarbitone. No direct comparison can be made but if the values obtained are recalculated (assuming the mice weighed 20 g) to correspond to Fig. 6 with injection rate in mg/kg/sec as the independent variable and threshold dose in mg/kg as the dependent variable, the result is similar to the one shown above. The injection rates varied between 0 1–1 6 mg/kg/sec. The lowest threshold was around 0.2 mg/kg/sec and with faster injection rates the thresholds increased. The increase

between 0.2 and 1 0 mg/kg/sec. was approximately 50 / as, compared with 20 / with the present method. This difference is difficult to explain merely by the difference in circulation time between rat and mouse It is probable that it depends on the inherent inaccuracy in McCANCE's method where the animal's reflexes were tested intermittently For the purpose of calculation, half the time interval between the reflex testings will be added to the circulation time resulting in an overshoot in time. This overshoot in time will add to the increase in threshold doses with higher injection rates It is thus probable that there is an optimal injection rate of hexobartitone in mice and that it is not much different from the one found in the rat

The present method to determine central nervous system sensitivity to hexobarbitone can be used for various purposes. The fairly constant threshold over several months in adult male rats makes it suitable for following changes in CNS sensitivity which may accompany long term treatment of various kinds.

Summary

A new method of studying the central nervous system sensitivity to a barbiturate is described. Hexobarbitone (enhexymal NFN) is infused continuously into a tail vein of the rat and the changes in the EEG are followed. The first appearance of a burst suppression with a duration of 1 sec. or more (silent second) is taken as a threshold and the dose needed to obtain this is used as a measure of the sensitivity The threshold dose is dependent on the infusion rate and there seems to be an optimal rate around 0.25 mg/kg/sec. The increase in threshold seen with faster infusion rates probably depends on the delay caused by the circulation time, before the hexobarbital reaches the central nervous system. With a rate of 0.25 mg/kg/sec. the circulation time seems to give an excess dose corresponding to 5–10 / of the threshold dose. The threshold dose is different between male rats but tends to be the same for the individual rat. The average normal threshold is approximately 65 mg/kg. No change in the threshold was seen over a period of 3–5 months.

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From the Department of Pharmacology University of Uppsala, Sweden
(Professor Ernst Bárány)

Hexobarbitone Sleeping Time in Rats Following Doses With Similar EEG Changes

By

Göran Wahlström

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A common method of determining the central nervous system depressant properties of a drug is to record the "sleeping time" which is in fact, the time when the righting reflex is absent. The drug to be evaluated can be given either in one dose or in several fixed doses to get a dose response curve.

In another paper (WAHLSTRÖM 1966) a threshold method for determining the central nervous system (CNS) sensitivity to barbiturates is described. The appearance of an electroencephalographic (EEG) criterion (burst suppression) after constant rate intravenous hexobarbitone infusion is used as a threshold. Shortly after the EEG-criterion has appeared the infusion is terminated. The dose infused is therefore close to the threshold dose.

In the present paper some aspects of sleeping times are presented following a threshold dose of hexobarbitone in otherwise untreated rats. These sleeping times are further more related to the sleeping times obtained in the usual way by a fixed dose, and the drawbacks of sleeping times as a measure of CNS sensitivity are discussed.

Methods

Male Sprague-Dawley rats were used except where otherwise stated. The initial weight of the rats was around 300 g before any experiments were performed. The rats were kept in a constant temperature room at 30°. The light in the room had a cycle of 12 hours of light and 12 hours of darkness. The light was turned on at 8 a.m. The rats had access to water and food pellets ad libitum.

The time without righting reflex was recorded automatically except where otherwise. This was performed on beds which measured 8 × 18 cm and were 5 cm high. The

bed there was a microswitch which was actuated when the weight of the rats pressed down on the bed. An impulse was passed through the microswitch every 10th second and was recorded on a counter The rats were placed on the beds either on their left or right side. When the rats regained their righting reflex they rolled over and more or less fell off the bed and the electric circuit was broken. The beds were automatically locked by a simple mechanical device consisting of a spring and a pawl which made it impossible to depress the beds again (if the rat were to crawl aboard again) and actuate the microswitch until the next recording was started by taking away the pawl. A brief description of the device has been given previously (WAHLSTRÖM 1958). It is essentially similar t the one described for mice by BOURA *et al.* (1965). The beds were placed in the temperature controlled rat room.

The observation of the sleeping time was also perf rmed in the temperature controlled rat room. The rats were placed on their backs at the beginning of the experiment. When they first rolled over they were gently placed back on their backs. The end point used was the bility of the rats to right themselves immediately (within 15–20 sec.) when placed on their back.

Two different kind of experiments were performed. In one hexobarbitone sodium (*enhexymal NFN — evipan* ®) *was injected intraperitoneally in a* fixed dose (100 mg/kg). Hexobarbitone was obtained in a powder form as the commercial preparation from Bayer AG Immediately before use it was diluted with distilled water to a concentration of 100 mg/ml and this sol ti n was injected. Sleeping time was taken as the time from the loss of the righting reflex to its return.

I the other experiments, hexobarbitone was nfused, intravenously at constant rate of 0.25 mg/kg/sec. if not otherwise stated. A dose of 150 mg/kg was taken from the stock solution described above, for each rat. This amount was further diluted with 0.25 sodium chloride to give a final volume f 1 ml. The volume rate was thus 0 1 ml/min. The EEG was recorded during the infusio as described elsewhere (WAHLSTRÖM 1966). When the fist *burst suppression of 1 second or more (the silent second) was noted* by the observer the infusion was stopped The infusion was actually stopped only at the end f 10 second increment f time, in which time zero was taken as the start of the infusion. The amount of hexobarbitone infused after the silent second could thus vary depending on whether the actual silent second was oticed and if there was sufficient time to stop the infusions by the *end of that 10 second time interval. Approximately 80 % of the infusions* were stopped within 20 sec. after the silent second (see F g. 4). In these experiments the end of the infusion was taken as the beginning of the sleep g time. The righting reflex had usually disappeared approximately 1 min. before th silent second (WAHLSTRÖM 1966). It usually took 3 min. to carry the rats t the beds the ratroom nd this time was included in the sleeping time.

Results

Comparison between directly observed and automatically recorded sleeping times

The sleeping times obtained by the automatic recording beds and by direct observation of the righting reflex were compared in spayed female Sprague-Dawley rats. The second sleeping time obtained *in* these rats was used Spayed *females* were used in this comparison as *they* have very long sleeping times and a more gradual return of *the* righting reflex as compared with males (see Fig. 1 series A) given the same dose of hexo-

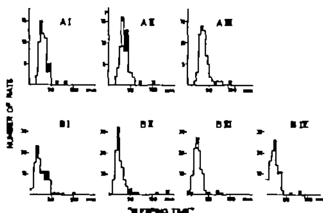


Fig 1 Frequency distribution of sleeping times fter hexobarbitone in male rats. In series A, 100 mg/kg was injected i. p. In series B hex barbitone was infused i. v to the silent second threshold. The same rats were used in all A (= 62) and all B (n = 110) experiments respectively Experiment I is the first one ever performed on these rats. The means (in min.) and standard deviations were in experiment A I 32.5 ± 11.3 A II 37 6 ± 13.0, A III 38.1 ± 14.0, B I 27 1 ± 15.3 B II 29 1 ± 20.0, B III 25 7 ± 11.9 and B IV 24.0 ± 12.2. Class intervals are 5 min. None of the rats showed zero sleeping time.

barbitone. Any difference between the two recording methods could thus be more easily seen

The experiments with the beds in one group of rats gave an average sleeping time (after 100 mg/kg i. p.) of 90 min and a standard deviation of 27 min (n = 43) The corresponding values in the other group which was directly observed were 109 ± 27 min. (n = 41) The bed method thus tended to show a return of the righting reflex earlier than direct observations. This was as expected, as the direct observations were based on an end point at which the rats had fully regained the ability to roll over when tested, while the beds recorded the return as the ability to leave the side position In spayed females it thus takes at least 15–20 min between the first appearance of the righting reflex and its complete return The uncertainties in both methods were, however approximately the same.

Comparison between the sleeping time after a threshold dose and after a fixed dose per kg body weight

In Fig. 1 the distribution of sleeping times obtained in two different manners is shown In series A hexobarbitone was injected intraperitoneally in a dose of 100 mg/kg. Three consecutive sleeping times were obtained in 62 male rats in the three experiments. The interval between the experiments was one week and the first experiment was the first one ever performed on these particular animals. The average induction times

which were not included in the sleeping times were in experiment A I 3.2 min. (range 1 0–12.5) A II 2.1 min. (range 1 0–10.0) and A III 1.9 min. (range 1 5–4 0)

In series B the sleeping time was obtained after a threshold determination ("silent second") The result of the threshold determinations in these rats have been published elsewhere (WAHLSTRÖM 1966, Fig. 4). In series B the dose of hexobarbitone was not fixed but depended on the appearance of the silent second and was thus individually determined.

Fig. 1 shows that in spite of the different method of injection and dose schedules used in the two series of experiments, the shapes of the distributions of the sleeping times were remarkably similar The mode is obvious in all distributions, even in series B where the doses given ranged between 45–105 mg/kg. Only in B I was there a tendency towards a wider range in sleeping times than in series A. The effect of the dose on the sleeping times in series B will be further discussed below

All the distributions in Fig. 1 are slightly skewed because some rats had extremely long sleeping times. In series A the mean in the first experiment was smaller than that of the others, but a corresponding difference was not seen in series B

The dose administered in the experiments in which the silent second was used as a threshold (series B) can be regarded as consisting of two parts. One part is the amount needed to obtain the silent second. The other is the amount infused after the silent second and this amount depends on the time when the infusion is stopped. Since the infusion was only stopped at 10 sec. time intervals after it had started, the experiments in series B can easily be classed according to the total amount of hexobarbitone infused.

Fig. 2 shows the amount infused after the silent second (excess dose) in relation to the total amount infused. It is clear from the figure that the excess dose was fairly constant over the range of doses given and that it usually amounted to 2–5 mg/kg.

In Fig. 3 the relationship between the dose of hexobarbitone infused and the sleeping time in series B has been further analysed The filled triangles in Fig. 3 are derived from a separate experiment. Two fixed doses of hexobarbitone (52.5 and 82.5 mg/kg) were infused in the same manner as in the threshold determinations. No attention was thus given to the silent second. The same rats got both doses, half of them receiving the lower dose first. These rats (series C) are not included among the rats used in series B.

Fig. 3 shows that in B II, B III, and B IV there was no dose response relationship between the amount of hexobarbitone infused and the sleeping time. In series C, infused with a fixed amount of hexobarbitone there was a clear relationship between dose and sleeping time. It has thus

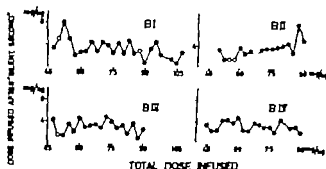


Fig. 2. The amount f hexobarbitone infused after the silent second had ppeared in relation to the total amount infused. This series is identical with series B in Fig. 1. The number of rats at each point is indicated by the symbols. Filled circle 10–16, half filled circle 5–9 and unfilled circle 1–4. Total number of rats 110 Average total dose infused and average dose infused after silent second were in experiment B I 76.3 and 3.3 mg/kg, B II 70.0 and 3.3 mg/kg, B III 69 8 and 3.3 mg/kg and in B IV 67 7 and 3.3 mg/kg.

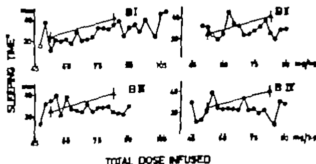


Fig. 3 The relation between total dose of hexobarbitone and sleeping time after continuous intravenous infusio in which the silent second was used as an indication to stop the infusion. This series is identical with series B in Fig. 1 and 2. The number of rats at each point is indicated by the symbols. Filled circle 10–16, half filled circle 5–9 and unfilled circle 1–4. Total number of rats 110. Triangles indicate experiments in which hexobarbitone was infused as in the B series, but with fixed doses without regard to the silent second (series C) The same rats (= 18) were tested with both doses. The bars indicate twice the s.e.m.

been possible to obtain the same sleeping time with a large range of doses of hexobarbitone provided that the silent second was used as an indication to end the infusion. This means that the barbiturate level necessary to give the silent second and the level just allowing for return of the righting reflex, must be fairly well correlated in this group of animals.

Matters were different in experiment B I In this experiment there seemed to be an increase in sleeping time with increasing doses of hexobarbitone ($r = 0.28$, $P < 0.01$) This increase runs roughly parallel to the

one obtained in series C, although the absolute values were somewhat smaller in B I. An attempt to explain this puzzling finding is given below

In experiments B II–IV the average variance in threshold dose was 76.97 (mg/kg)^2 and in sleeping time it was 231.52 min^2 The corresponding figures for B I were 169 06 and 233 11 The factors causing the increased variance in threshold dose in experiment BI had thus no detectable influence on the variance of the sleeping times. Since the return of the *righting reflex* (sleeping time) was not affected by the extra factors influencing the threshold dose in B I the larger doses in B I compared with the other B experiments must give a dose response. The unknown factors in B I which caused the increase in threshold were presumably related to the unfamiliarity of the test situation in the first experiment (WAHLSTRÖM 1966)

Influence on the sleeping time of hexobarbitone doses deviating from the threshold dose

In Fig. 3 it was shown that in experiment B II–B IV all the doses infused gave approximately the same sleeping time, when the silent second was used to determine the end of the infusion. It was therefore of interest to see how the amount of hexobarbitone influenced the sleeping time when the dose was related to the dose needed to produce the silent second. This is shown in Fig. 4

Two series of experiments were done, in which two fixed doses of hexobarbitone were infused in the same way as in the threshold determination. The same rats were used for both doses. In series C (included in Fig. 3) the fixed doses were 52.5 mg/kg and 82.5 mg/kg, and in series *D* with another group of rats, they were 45.0 and 52.5 mg/kg. Previous to the fixed dose experiments, the threshold determination of each rat that had the smallest excess dose (amount infused after the silent second) was selected out of 2–3 determinations. The first determination performed in the rats was not used These selected threshold determinations are designated as the selected experiments. The dose needed to obtain the silent second in each rat was determined from the selected experiments. Because of the selection, the dose which gave the sleeping time was very close to that needed to produce the silent second.

Also shown in Fig. 4 are the sleeping times in the second determination performed in 502 other rats. The rats in series B were included but not those in series C and D The rats were grouped according to the dose infused after the silent second (the excess dose) and the material was classed with a hexobarbitone excess of 2 mg/kg as class width from 0 up to an excess of 10 mg/kg. Above 10 mg/kg all rats were classed together

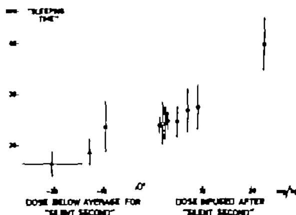


Fig. 4. The effect of the dose infused immediately before and after the silent second on the sleeping time. Averages are plotted and those below the silent second are expected and not true values.

Squares denote series C. Fixed doses of 52.5 and 82.5 mg/kg were infused as in the usual threshold determination (filled squares). Selected threshold experiment with small excess dose is denoted by open square (see text). The average doses needed to obtain the silent second were in the 82.5 mg/kg experiment 60.1 mg/kg and in the selected experiment 62.1 mg/kg. The range of the excess dose was 7.8–35.4 and 0.5–3.9 mg/kg respectively. The selected experiment was used to calculate the dose deficit in the fixed dose experiment with 52.5 mg/kg. The same rats were used in all experiments (n = 18).

Triangles denote series D. Fixed doses of 45 and 52.5 mg/kg (filled triangles) and selected experiment (unfilled triangle) were obtained as in series C. The dose needed to obtain the silent second in the selected experiment was 65.4 mg/kg. The range of the excess dose was 0.8–6.5 mg/kg. The same rats were used in all experiments (n = 19).

Unfilled circles denote the material of the second threshold determination in 502 rats. The material was grouped according to mg/kg infused after the silent second and then classed. The central point in each class was weighted. The class width and the number of rats in each class were 0–2 mg/kg 207, 2–4 mg/kg 178, 4–6 mg/kg 45, 6–8 mg/kg 29, 8–10 mg/kg 27 and more than 10 mg/kg 16.

The bars indicate twice the s.e.m. The horizontal bar at the lower left is applicable to all points below the silent second.

The class means with regard to excess were weighted as there were different numbers of rats in each group in the class.

Fig. 4 shows that the slope of the dose response curve tends to increase with higher doses. This general shape agrees with the expected time course of concentration in the CNS. It is probable that the first part of the concentration decay curve shows a faster fall than the later parts. With larger doses, the CNS level of barbiturate which must be passed for the righting reflex to reappear could not be reached on the rapidly falling part of the concentration curve and a small increase in dose would give a larger increase in sleeping time.

In many experiments an arbitrary limit was chosen, above which the

sleeping times of the rats were discarded since they were given too much hexobarbitone after the silent second (excess dose). 7.5 mg/kg was taken as the limit. As seen in Fig. 4 this excess would correspond to an increase in sleeping time of about 3 min.

It is also of interest to see to what extent the observed variability in sleeping time is caused by the variable excess. A good estimate can be obtained by using the fixed dose of 82.5 mg/kg and the selected experiments in series C. In the fixed dose experiment the range of the excess was approximately 25 mg/kg and the standard deviation of sleeping time 10.9 min. In the selected experiments in which the range of excess is 3.4 mg/kg and which is situated on the flatter part of the dose response curve, the standard deviation of sleeping time is 9.1 min. Thus, the extra variability caused by allowing a limit of 7.5 mg/kg for excess dose is very small.

Some properties of the sleeping times after hexobarbitone infused to obtain the silent second

Table 1 shows an experiment in which 9 threshold determinations were performed in 7 rats to obtain the silent second. The first determination was discarded and the others followed at an interval of 2–3 days. After each threshold determination the sleeping time was recorded. One sleeping time had to be discarded as more than 7.5 mg/kg hexobarbitone was infused after the silent second. An analysis of variance was performed with one missing figure according to SNEDECOR (1956). No difference in sleeping time could be established between the different days and there was only a suggestive difference between individual rats. The results of the threshold determinations have been published elsewhere (WAHLSTRÖM 1966).

Table 1

Analysis of variance in 8 sleeping times after threshold doses in 7 rats (one missing figure).

Source of variation	DF	Mean square	F	P
Rats	6	204.55	4.99	<0.05
Days	7	17.04	0.4	>0.05
Residual	41	40.99		

Table 2

Long term changes in sleeping times after threshold doses.

Experiment	Average pre-experimental: Sleeping time min.	Average pre-experimental: Weight g	Number of rats	Time interval without experiment. Months	First determination after the interval: Change in sleeping time from pre-experimental verage. Per cent: Mean	First determination after the interval: Change in sleeping time from pre-experimental verage. Per cent: s. e. m.	Weight at A
I	41 1	403	9	3.3	84.7	8.7	477
II	28.8	358	11	4 0	100.5	8.4	461
III	4.8	376	6	4.7	90.6	9.5	486

The long term changes in sleeping time after a threshold dose are shown in Table 2. In no case was more than 7.5 mg/kg infused after the occurrence of the silent second. The pre-experimental level was calculated on the average of 3 or on a few occasions, only 2 determinations. After these had been performed the rats were left undisturbed for 3–5 months. The sleeping times recorded in the first experiment after these months have been calculated as per cent of the pre-experimental value. Table 2 shows that in experiment I and III there was a small and doubtful tendency towards a decrease in the sleeping times. There were no changes in the doses needed to obtain the silent second in the three experiments (WAHLSTRÖM 1966) It is possible that weight and/or age could be responsible for a tendency towards smaller sleeping times.

The sleeping times obtained in experiment BII, BIII and BIV (in which approximately the same amount of hexobarbitone were infused) have been divided into classes depending on the time after the onset of light in the rat room when the determination was performed. The results are shown in Fig. 5 There was a tendency towards shorter sleeping times in the first few hours after the light was turned on and before the light was turned off. No sharp maximum was, however found. There were no corresponding changes in the amount needed to obtain the silent second (WAHLSTRÖM 1966) The slight tendency towards a diurnal variation can be explained by a change in metabolism due to the known rhythmic in the function of the liver (KLEITMAN 1963 chap 17 HALBERG 1960) As the effect of metabolism on the fairly short sleeping times obtained after the threshold dose is presumably slight, no more than a slight tendency towards an influence of the light time could be expected.

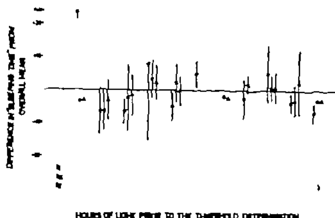


Fig. 5. The influence of hours of light before the silent second threshold determination on the sleeping times. Experiments B II, B III, and B IV (Fig. 1) have been used. Differences between the average sleeping times obtained i each hour after the light was turned on and the overall mean for the corresponding experiment have been calculated. The number of rats at each point and the symbols used are shown in the lower part of the figure. The bars indicate twice the s.e.m.

Effect of different infusion rates on the sleeping time after a threshold dose

In Fig. 6 the change in sleeping time is plotted (in per cent) against different infusion rates (obtained by changes in volume rate) The experiments are the same as those used previously (WAHLSTRÖM 1966) to investigate the effect of different infusion rates on the dose of hexobarbitone, needed to obtain the silent second. All experiments, in which more than 7.5 mg/kg hexobarbitone was infused after the silent second, were excluded. It is evident from Fig. 6 that the *standard rate* (0.25 mg/kg/sec.) gave the shorter sleeping time. The pattern of the changes, with higher and lower infusion rates, of the sleeping times are closely parallel to the patterns of changes in the threshold dose (WAHLSTRÖM 1966) There is a slow gradual increase with higher rates and a steeper rise with slower rates

Because of the similar changes, it was of interest to see whether an increase in dose due to "slower than standard rate" caused the same increase in sleeping time as a corresponding increase in dose due to "faster than standard rate It is clearly evident from Fig. 7 that an increase in dose due to a faster infusion rate was more effective in increasing the sleeping time, than a corresponding increase in dose due to a slow infusion rate. It is probable that the increase in dose to obtain the silent second with faster infusion rates is caused by the amount of hexobarbitone which has still not reached the CNS when the silent second appears (WAHL-

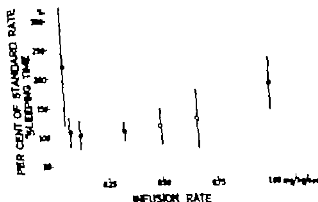


Fig. 6. The effects of different speeds of infusion on the sleeping times obtained after silent second threshold determinations. The concentration of hexobarbitone was kept constant. Filled circle denotes standard rate (0.25 mg/kg/sec.). The standard rate sleeping times and number of rats from which the percentages have been calculated are shown below. All rats with an excess of more than 7.5 mg/kg over the silent second dose were excluded. The bars indicate twice the s.e.m.

Rate	1.0	0.67	0.5	0.33	0.125	0.08	0.06	mg/kg/sec.
Sleeping time	35.8	36.2	29.6	35.4	33.7	30.9	26.7	min.
	8	9	11	16	11	13	11	

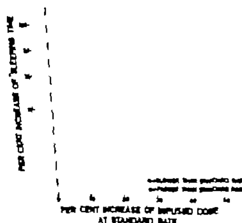


Fig. 7. The effect of an increase in hexobarbitone dose due to slower or faster than standard rate infusions (0.25 mg/kg/sec.) on the sleeping times. The same experiments as in Fig. 6 have been used.

STRÖM 1966). Thus the extra dose which has not yet reached the CNS when the infusion is stopped should produce a larger increase in sleeping time, than an extra dose which has already been redistributed or perhaps metabolized due to the slow infusion rate.

Discussion

Sleeping time is commonly defined as the time when the righting reflex is absent. Actually there are two events which are recorded: loss and return of the righting reflex. There are thus three possible times which could be measured. The time from the administration of the drug to the return of the righting reflex (*return time*), the time before righting reflex is lost (*induction time*) and the difference between these two times, i. e. the sleeping time. That these three times ought to be carefully considered in experiments using sleeping times is exemplified by series A (Results). The average sleeping time in experiment AI is 5.1 min. shorter than the corresponding time in experiment AII (Fig. 1). The probability that this difference is due to chance is less than 0.05 (Student's t). It thus could be concluded that the rats consistently wake up earlier after the administration of the first dose. A closer analysis of the data reveals, however, a small but significant difference (1.1 min.) in the induction time between AI and AII ($p < 0.01$). The larger difference (4.0 min.) in the return times was not significant. The consistent difference between experiment AII and AI was thus due to a longer induction time in experiment AI.

A change in induction time is not equivalent to a change of the same magnitude in return time, when CNS sensitivity to hexobarbitone is studied. Such a comparison must be based on the corresponding changes in brain concentrations of hexobarbitone. The brain concentrations of hexobarbitone in mice after intraperitoneal injection have been carefully studied by WINNE (1964). Calculated from his figures, the change in brain concentration in mice was approximately 15–20 times faster at the approximate time of loss of the righting reflex, than at the time of return of the reflex. Since the situation must be similar in rats, a change in CNS sensitivity should cause much larger changes in return time than in induction time. The 1.1 min. difference in induction time between AI and AII thus very probably represents more of a true CNS difference than the 4 min. change in return time between the experiments. The cause of the increased induction time in the AI experiment presumably is related to the unfamiliarity with the test situation, in the same way as in the threshold experiments.

When regarded as a measure of sensitivity of the CNS, small changes in the sleeping times could thus either mean a large change in sensitivity, i. e. if induction time was influenced, or virtually no change, i. e. if return time was influenced. The changes obtained in induction time are, however, small and tend to be negligible if more marked changes are obtained in return time. In most experiments with sleeping times, return times are thus responsible for the change measured.

The event "return of righting reflex" depends only on the sensitivity of CNS to the inducing drug. The return time however also depends on the rate of disappearance of the drug. This disappearance is determined by the metabolism and/or the redistribution of the drug. Return time thus is influenced by at least three factors of which CNS sensitivity is only one.

Several examples can be given as illustration of the effect of metabolism of the inducing drug on sleeping time increased metabolism of the inducing drug due to a previous administration of the same or related drugs (REMMER 1962 CONNEY 1960) changed metabolism due to administration of a different drug (FUJIMOTO *et al* 1960) and changed metabolism due to the sex hormones (REMMER 1958 a) or glucocorticoids (REMMER 1958 b)

The effect on sleeping time of the internal temperature of small animals can be explained by a changed metabolism (MORRIS 1963 a FUHRMAN 1947) However temperature also seems to have a direct effect on CNS sensitivity (WINNE 1964)

If sleeping time measurements are made with a drug in which re distribution contributes to the termination of the action then the long term experiments will be difficult to evaluate if the body weight changes. As lean tissue such as muscle seems to be the first compartment into which short acting barbiturates are redistributed (GOLDSTEIN & ARONOW 1960 PRICE *et al.* 1960) changes in weight due to increased muscle mass could decrease the sleeping times. Changes in weight due to other reasons could increase them. An example of a regression of sleeping time on body weight after pentobarbitone administered in mg/kg doses is given by MORRIS (1963 b). Whether this was due to changes in re-distribution or metabolism can not be deduced from the sleeping time data given. In the present experiments, an effect of weight on the sleeping times can not be excluded in some threshold experiments (for instance Table II) A slightly significant correlation between weight and sleeping time was, however found only in experiment BIV ($r = 0\ 19$ $p < 0.05$ $n = 110$), but not in the other B experiments. However changes in metabolism with age or weight can not be excluded and the observations are not necessarily explained by re-distribution alone.

The sleeping times are also influenced by hydration and dehydration (BROZELLECA & MANTHEI 1957 RAMWELL & LESTER 1961) The solvents in which the barbiturate is administered i p and the dilution effect of peritoneal fluid which presumably could influence the absorption are still other factors which should be considered (RÜMKE & DE JONGE 1962 SMITH 1961) Even when as many external factors as possible are rigorously controlled and analysed there is a considerable variation between sleeping times of control groups treated in the same manner but participating in different experiments (RÜMKE *et al* 1963).

Sleeping time measurements are thus influenced by a multitude of factors which are hard to evaluate without a detailed knowledge of the distribution and metabolism of the barbiturate under study Most of these factors influence the return time. If CNS sensitivity is to be studied, sleeping time measurement is a rough and uncertain method. When an experimental variable is introduced, all other possible influences on the sleeping times must be excluded before changes in CNS sensitivity can be ascertained

Fig. 4 shows that there is a positive correlation between the amount infused after the silent second and the sleeping times obtained. This means that at a fixed dose of hexobarbitone, rats with low threshold doses will tend to get long sleeping times and rats with high threshold doses will tend to get short sleeping times. When the threshold dose for the silent second is used to terminate the infusion, then there is no relation between sleeping time and threshold dose in the B series (Fig. 3) except in BI Under normal conditions sleeping times and threshold dose are thus indices of sensitivity to hexobarbitone in the CNS and are related to one another But the silent second is less affected by extracerebral factors.

Summary

A method for the automatic recording of "sleeping times" in rats is described In spayed females after 100 mg/kg hexobarbitone (enhexymal) injected intraperitoneally this method gives sleeping times which are approximately 20 min. shorter than those observed directly but the standard deviations are the same with both methods.

Sleeping times in two groups of male rats were recorded after different treatments.

1 The rats got 100 mg/kg hexobarbitone intraperitoneally

2. Hexobarbitone was infused continuously intravenously at a rate of 0.25 mg/kg/sec. and the dose needed to obtain a predetermined EEG-criterion (the silent second) was used as an indication for ending the *infusion.*

Similar distributions of the sleeping times were obtained in the intraperitoneal group (3 successive experiments) and the intravenous threshold group (4 successive experiments) In the threshold group there was no dose response relationship (range of doses 45–90 mg/kg) with regard to sleeping time, except in the first experiment. In this experiment the threshold doses were also increased. A corresponding change was found in the first intraperitoneal experiment in which the induction time was increased.

A dose response curve plotted with doses related to the amount needed

to obtain the silent second revealed a gradually increasing slope with higher doses.

If the infusion rates of hexobarbitone were changed from the standard rate 0.25 mg/kg/sec., the sleeping times increased. This increase was similar to the increase in threshold dose found earlier with changed rates. An increase in dose due to a faster than standard rate, increased the sleeping times more than a corresponding increase due to slower than standard rate.

The disadvantages of sleeping times as a measure of central nervous system sensitivity to drugs are discussed.

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From the Department f Obstetrics and Gynaecology University Hospital, Lund, Sweden (Professor Alf Sjövall)

Effects of Vasopressin on Uterine Blood Flow in the Rabbit

By

Anthony M. Carter Peter J Lewis and Lars Philip Bengtsson

(Received April 29 1966)

It is well known that the posterior pituitary hormone vasopressin has an effect on certain vessels. The action of the drug on the uterine vasculature was first investigated in pregnant cats and spayed cats under steroid treatment by Robson & Schild (1938) Using a constant rate perfusion method they showed that the injection of vasopressin (0 05–0 1 IU) into the uterine artery led to an increase in perfusion pressure, indicating vasoconstriction In a small number of experiments they also found that vasopressin (0 05–0.2 IU i. v) caused a decrease in uterine volume as recorded with an oncometer It has also been demonstrated that vasopressin (0 01–1 0 IU) administered via the uterine artery of pregnant and portpartum bitches causes a reduction in flow through the vessel of between 30 % and 100 % as measured by an optically recording rotameter (Ahlquist & Woodbury 1947 Ahlquist 1950)

Large doses of vasopressin given to pregnant animals have been shown to cause foetal death in the rabbit (Bengtsson 1957), rat (Byrom 1937) and mouse (Parkes 1930) This effect was first demonstrated in the rabbit by Knaus (1926) using posterior pituitary extract (2 mg moist gland extract, i. v) Since he failed to obtain a response before the 18th day of pregnancy and it is at this point that the placenta becomes separable from the uterine wall the effect was attributed to the oxytoxic properties of pituitrin (Huggett & Hammond 1952). Bengtsson (1957) has, however shown that the active moiety is vasopressin oxytocin being without any effect

It is not known if the deleterious effect of vasopressin on the foetus is related to its effect on the uterine vasculature. This question is being studied in our department and the present paper deals with the effect of vasopressin on uterine vein flow in pregnant and nonpregnant rabbits.

The rate at which blood traverses the uterus of the rabbit was first investigated by BARCROFT, HERKEL & HILL (1933) using extensive dissection and collection of the diverted venous outflow. Search of the literature fails to reveal any subsequent attempt to measure uterine blood flow in this animal or any information on the pharmacological responses of the vascular musculature in the rabbit uterus. For the purpose of the present study we have developed a relatively simple technique which enables continuous monitoring of uterine vein flow in the rabbit.

Methods and Materials

Animals

Swedish white Land rabbits were used in this study. The mean length of gestation in this strain is about 31 days. They were treated in two groups of six animals, pregnant and nonpregnant, with mean weights of 3.4 kg and 3.3 kg respectively. The first group was artificially inseminated with diluted semen from fertile males and given chorionic gonadotrophin (gonadex ® Leo, 33 IU i.v.) to induce ovulation; the experiments were performed 26 days later. The second group consisted of nonpregnant, parous animals.

Experimental

Tracings of uterine vein flow and mean carotid arterial pressure were obtained on smoked paper by the following procedure. The animal was anaesthetised with pentobarbitone (mebumalnatrium 6 %, 40–50 mg/kg, i.v.). Percutaneous tracheotomy was performed. The left external jugular vein was cannulated and connected to a polyethylene burette which served as a blood reservoir. Heparin (1000 IU/kg) was injected via the catheter and washed in with 2 ml physiological saline. The left common carotid artery was cannulated and connected by means of saturated sodium sulphate solution to a mercury manometer pressure recorder.

The bdomen was opened by a midline suprapubic incision. The bladder was emptied by digital compression. In this way the middle third of the vagina was exposed with the supporting broad ligament on either side. The peritoneum of the broad ligament was carefully broken immediately lateral to the vagina, at a point 1 cm craniad to the neck of the bladder nd the uterine vessels were cleared of supporting tissue for about 1 cm.

The terine artery and vein vary greatly in their mutual relations (BARCROFT & ROTHSCHILD 1932, BARCROFT, HERKEL & HILL 1933), the artery usually running over the dorsal aspect of the vein and being bound tightly to it. More rarely the artery and vein are entirely separat or the artery is along within the lumen of the vein in form of mesarterium, resembling the arrangement of a tendon within a synovial sheath. The operative procedure therefore required great care in the separation of artery from vein. When this h
achieved, polyethylene catheter was inserted into the vein nd tied in.
was available, to enable selection of the largest size which it was possible
practise only tw sizes were needed one for the nonpregnant animals (
1.14 mm) and nother for the pregnant animals (e. d. 1.90 mm, i. d. 1
of the catheter was connected to the dropping chamber of phot
The apparatus was so arranged that blood flowed along the
ber at the same level as it l ft the vein. Each drop falling thro
as deflection of a kymograph tracing. The blood was coll
and returned to the jugular vein from the blood reservoir
constant by adjusting the inflow tap.

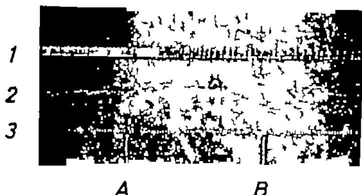


Fig. 1 Part of typical kymograph record. 1 Uterine vein flow each deflection of the tracing indicates passage of drop of blood through the counting chamber 2: Mean carotid arterial pressure. 3 Time marked every sixth second. An injection of 1.0 IU vasopressin was made between A and B. Nonpregnant rabbit.

Vasopressin

The vasopressin used in this study was synthetic lysine-vasopressin (postacton ®, Ferring) with pressor activity of 166.7 IU/mg and an oxytocic activity of 2.5 IU/mg.

Various preliminary experiments were performed to ascertain the most suitable method of administration. Several points emerged from these. Firstly marked tachyphylaxis and persistence of action were observed which rendered repeated bservations on the same animal unreliable. Secondly rapid injections led to rapid and violent fluctuations in the blood pressure. Thirdly there was marked variation in the individual response to the drug. On consideration of these factors it was decided to administer the drug as slow injection and to give only one dose to each animal, namely 1.0 IU The injection was made through a fine polyethylene catheter inserted into the jugular cannula, using an infusion pump which delivered 0.2 IU/min. for five minutes.

Results

Part of a typical kymograph tracing is shown in Fig. 1 This illustrates the effect of vasopressin injection on mean carotid arterial pressure and uterine vein flow Twelve such records were obtained, half from nonpregnant animals and half from animals on the twenty-sixth day of pregnancy From these records the uterine vein flow was calculated in drops per minute for each of the following five minute periods 1) immediately before injection 2) during injection 3) immediately after injection 4) 20–25 minutes after injection. A typical histogram based on such an analysis is shown in Fig. 2.

For the purpose of comparison, the figures thus obtained were converted to percentages of resting flow defined as the average flow during the five minutes before drug administration These percentages are given in Table 1 Means of the percentages were then calculated for the nonpregnant and



Fig. 2. Uterine vein flow in drops per minute plotted against time in minutes. The horizontal bar indicates an injection of 1.0 IU vasopressin. Rabbit on 26th day of gestation.

Table 1

Uterine vein flow expressed as a percentage of resting flow for each minute of the analysis. See text for further explanation. In each case vasopressin was injected at a rate of 0.2 IU/m during minutes six to ten.

Minutes	Nonpregnant animals 1	2	3	4	5	6	mean	Pregnant animals 7	8	9	10	11	12	mean
1	96	101	103	98	102	105	101	94	94	102	97	101	92	97
2	105	98	103	98	104	99	101	103	102	106	102	97	97	101
3	102	103	103	101	99	99	101	107	96	95	104	101	103	101
4	102	101	91	98	99	99	98	90	104	98	97	101	104	99
5	96	98	99	104	97	99	99	107	104	98	100	101	104	102
6	88	98	87	101	79	92	91	107	106	91	93	94	101	99
7	37	77	37	81	32	46	52	81	94	63	82	81	95	83
8	9	65	12	67	15	20	31	56	92	59	65	71	81	71
9	6	60	0	64	12	20	27	51	98	51	20	68	74	60
10	11	60	0	61	10	13	26	51	100	51	26	62	72	60
11	11	60	0	61	10	13	26	64	98	51	24	52	67	59
12	11	62	0	61	10	7	25	56	102	51	26	33	68	56
13	11	65	4	64	10	7	27	60	100	51	24	33	68	56
14	14	69	0	58	10	7	26	68	102	59	28	42	63	60
15	17	67	0	61	10	7	27	73	100	59	15	62	68	63
30	23	79	12	78	17	13	37	–	96	75	69	58	72	74
31	28	81	12	78	22	20	40		88	75	69	42	76	70
32	25	84	17	58	25	13	37	–	90	75	58	58	70	70
33	25	84	17	75	22	33	43	–	92	71	63	52	59	67
34	25	86	17	78	22	40	45		90	75	63	55	76	72

pregnant groups respectively. These are also given in Table 1 and are plotted against time in Fig. 3.

This analysis of the results allows of the following observations.

In all the nonpregnant animals and in five of the pregnant animals, administration of vasopressin led to a marked reduction in uterine vein flow (Table 1 and Fig. 3).

The extent of the individual response varied in both groups.

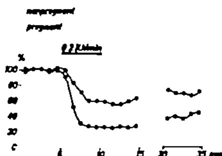


Fig. 3. Uterine vein flow as percentage of resting flow for pregnant and nonpregnant groups (means) plotted against time. The horizontal bar indicates injection of 1.0 IU vasopressin.

The reduction in blood flow first became marked during the second minute of the injection and it usually became maximal towards the end of the injection.

In no case was complete recovery to the resting flow level observed. One animal was followed for 1½ hours after the injection without full recovery of flow (Fig. 4)

The reduction in blood flow bore no clear relation to the pressor response, which was marked in all cases. There was always a rapid rise in mean carotid arterial pressure during the first minute of the injection, whilst the uterine vein flow remained at or near the resting level In the single experiment where no reduction of flow occurred, there was nevertheless a pressor response of 24 mm Hg. In all cases the pressure returned to values at or near the resting level after the injection, but there was no corresponding recovery of blood flow (cp Fig. 4)

The mean reduction in flow was greater in the nonpregnant than in the pregnant group (Fig. 3) We have attempted to assess the significance of this difference by performing a suitable t-test for the difference between



Fig. 4. Uterine vein flow (histogram) and mean carotid arterial pressure (upper curve) plotted against time. The horizontal bar indicates an injection of 1.0 IU vasopressin. Nonpregnant rabbit.

means of two small samples. The results indicate that the observed difference is probably significant during the period of injection but not subsequently The apparent discrepancy between this conclusion and the trend of the curves in Fig. 3 depends on the fact that, as the two sets of means diverge, the individual variation in response within the two groups also increases. Further investigation of the question would require much larger samples, allowing the application of a more powerful test.

Discussion

The results presented above indicate that vasopressin has a marked effect on the uterine blood flow of the rabbit in the dose administered here. This result is in accordance with the previous work carried out in cats and dogs (ROBSON & SCHILD 1938 AHLQUIST & WOODBURY 1947 AHLQUIST 1950) suggesting that the effect may be common to a wide range of mammals.

The reduction in blood flow appeared to be sustained, even when the arterial pressure had returned to and was maintained at a value close to the resting level (cp Fig. 4) Since complete recovery to resting flow levels was never observed it is, however difficult to eliminate the possibility that this sustained reduction was an artefact depending on some aspect of the technique used It is therefore of interest that experiments in sheep, where an electromagnetic flowmeter was employed, have shown reduction of arterial flow for 100 to 120 minutes following intravenous injection of oxytocin, which is a close chemical relative of vasopressin (ASSALI, DASGUPTA & KOLIN 1959) Prolonged action of vasoconstrictor drugs on the uterus was also reported by AHLQUIST (1950), who attributed this to trapping of the drugs in the constricted vascular bed.

The marked reduction of uterine blood flow observed following vasopressin treatment must necessarily interfere with placental blood flow Whether this is the cause of the foetal death reported by various investigators (BENGTSSON 1957 BYROM 1937 PARKES 1930) is at present under investigation.

It seems unlikely that the observed reduction in blood flow depends on a general systemic effect since it appears to be maintained. Thus a reduction in cardiac output for such a prolonged period could be expected to lead to circulatory collapse. A reduction in uterine blood flow due to local action may be mediated through mechanical constriction of the blood vessels due to myometrical contraction as well as by active vasoconstriction. Results obtained with oxytocin, which is a more powerful myometrial stimulant than vasopressin, would seem to rule out myometrial contraction as the operative factor Thus AHLQUIST (1950) found

that a greater reduction of uterine blood flow occurred with vasopressin than with oxytocin, which sometimes caused an increase in flow

If vasopressin causes active vasoconstriction within the uterine vascular bed, it is pertinent to enquire whether these vessels are particularly sensitive to vasopressin or whether their response is a manifestation of the general vasoconstrictor properties of the drug In this respect it is notable that we found a complete dissociation of the reduction in uterine vein flow from the general pressor response of the animals as recorded from the carotid artery The rise in arterial pressure was rapid and usually became maximal before any effect on uterine venous flow was noticeable. Furthermore the pressure always returned to values near the resting level after completion of an injection, whereas the reduction in flow appeared to be sustained. The possibility that different responses to vasopressin occur in different organs is given support by isotope studies, which indicate that lysine-vasopressin causes a redistribution of blood amongst the organs of rats (BERDE 1965) This concept also seems to be confirmed by radio-angiographic studies in dogs (ARONSEN & NYLANDER 1964)

The reduction of uterine venous flow demonstrated was obtained with relatively high doses of vasopressin. The effect of lower doses and the effect of the drug on the human uterine vasculature should be carefully investigated. If small doses of vasopressin prove to influence blood flow through the human uterus and placenta, this may have various implications. Firstly it would act as a warning against the use of vasoconstrictor drugs in the treatment of maternal hypotension during pregnancy (GABEL, ROMNEY & KANDORA 1961) Secondly changes in placental blood flow may interfere with placental functions, including the production and transport of hormones, and thus influence the endocrine control of pregnancy and parturition.

Summary

A technique is reported for continuous monitoring of uterine blood flow by diverting the outflow from one uterine vein through the dropping chamber of a photoelectric flowmeter The technique was applied to an investigation on the effect of lysine-vasopressin on uterine blood flow in pregnant and nonpregnant rabbits. It was found that the drug evoked a marked reduction of flow in both groups, which appeared to be sustained over a long period. The temporal development of this response exhibited a pattern different from that of the pressor response as recorded from the carotid artery The significance of these results is discussed.

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From the Pharmacological Laboratory of Dumex Ltd.,
Copenhagen, Denmark

The Embryotoxic Effect on Rabbits of Monophenylbutazone (Monazan (®)) Compared with Phenylbutazone and Thalidomide

By

Valdemar Larsen and Erik Bredahl

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Even though phenylbutazone has been used clinically since 1949 (BELART 1949) and monophenylbutazone since 1959 (A. LANDI), only a few publications seem to deal with the embryotoxic effect of these drugs. TRIEBOLD *et al* (1957) have, however found no reduction in fertility in experiments with female rats which were given 33.3 mg/kg phenylbutazone subcutaneously 8 times within the first 18 days of pregnancy

The present investigation is very similar to an investigation of "The Teratogenic Effects of Thalidomide, Imipramine HCl and Imipramine-N Oxide HCl on White Danish Rabbits" (LARSEN 1963) At the same time, it is an investigation of embryotoxic effects in general, and of a possible relation to fertility

Materials and Method

Experimental animals and diet

The white Danish rabbits used came from the Danish State Serum Institute and all had previously had from 2 to 11 litters. They were divided into 6 identical groups according to the number of previous litters. The males used for mating were also white Danish rabbits. Both males and females were fed on hay oatmeal, carrots as well as Barre's mangold ad libitum. They were not given water

Drugs investigated and method of administration

For comparison with monophenylbutazone we used the clinically well-known compound phenylbutazone as positive control there was thalidomide group, and as negative control group 8 rabbits who were given the same amount of diluent (water with 3 % glucose) as used for the other groups and administered in the same ay see below Phenylbutazone was used as drug of reference, since it is very similar chemically and has the clinical indications.

30•

Phenylbutazone was used in the form of vials containing a sterile filtered 2.5% solution with a pH of bout 8, mad in our pharmaceutical laboratory from Phenylbutazone (in conformity with the tests of the B. P 1963)) with N-NaOH These vials were kept in the dark and were used for the full duration of the experiment. Monophenylbutazone was used in the form f vials obtained from the original packages "Monazan ® Dumex, vials each containing 3 ml (Danish Speciality No. 2567) numbered 40816 K. Each ml contained 0.150 g 1-phenyl-4-butyl-3,5-dioxopyrazolidine" Both kinds of vials were first opened immediately before use and diluted with a sterile 3 / solution of glucose. At the end of the period of injection, any remaining vials of phenylbutazone and monophenylbutazone were examined by our analytical laboratory Of the declared contents, 99.0% of the monophenylbutazone and 97.2 of the phenylbutazone were found. We thus made sure that the animals received the intended amount of the agents, since this is important when dealing with labile compounds.

These two substances were, given together with as much diluent as 10 ml/kg per rabbit. This was done to prevent tissue damage (RECHENBERG 1961 p 85) and to prolong the absorption time and the period of action

The thalidomide used, was the same as in previous experiments thalidomide P2 10/7 49, a gift from Distillers Co (Biochemicals) Ltd Liverpool. A suspension containing 60 mg/ml was given in a dose of 1 ml/kg rabbit. We deliberately injected thalidomide as well as *phenylbutazone and monophenylbutazone* subcutaneously into the back. It is ell-known, that thalidomide has a teratogenic effect, when given subcutaneously A further advantage of the subcutaneous rout as opposed to the oral or intraperitoneal route is the elimination of a possibl local effect on the embryos and on the stomach of the dam, so that any effect produced on either of these must be ascribed to a transport of the agents by the blood stream.

Dosage

According to ADAMI (1956) the LD50 of phenylbutazone given subcutaneously to rabbits is *270 mg/kg theref re doses of 60 mg* and 30 mg/kg rabbit given daily are approximately $\frac{1}{4}$ and $\frac{1}{10}$ respectively of the LD50. The drugs were administered for the first 20 days (including Sundays) fter mating but not on the day of mating *itself* This long period was chosen, as we not only wished to investigate the teratogenic effect but also the embryotoxic effects in general compare in this respect with the considerations f MCCOLL *et al.* (1965). The doses of monophenylbutazone were 60 mg, and 150 mg/kg per day and of thalidomide, it was also 60 mg/kg

Experimental procedures

Table 1 gives a survey of the experiment and the results with regard to the umber of live-born young their weight and variations in weight. Only a few of the dams were allowed t give birth naturally in the great majority Caesarean section was performed so as to enable us to btain information regarding dead foetuses, *as* well as of retention of parts of either foetus or placenta in the uterus. The young so removed were allowed to *live for* bout 1 hour fo gross examination they were then killed with ether marked, weighed, grossly examined internally and finally examined for keletal anomalies.

Examination for skeletal anomalies

This was *performed* in two ways which supplement each other 1) radiography and 2) staining of the skeleton with alizarine in alkaline solution. The latter method was described by DAWSON (1926) the soft tissues were made partly transparent by treatment with

a weak solution of sodium hydroxide followed by a graded series of glycerol with decreasing concentrations f water while even very small parts of the skeleton were stained red by alizarine dissolved in the aqueous liquid. These stained bone preparations will here be named Dawso preparations. A rule only no radiogram was taken of the individual animals in side projection and the skeleton was examined by looking for the following bones in the *radiograph*

1) scapula, 2) clavicle, 3) humerus, 4) radius and ulna, 5) pelvis (4 bones), 6) femur 7) sternum as dotted line, 8) cervical segments (7), 9) thoracic segments (12), 10) lumb *segments (7), 11) sacral segments (4), 12) outline of skull with upper nd lower jaw nasal* bone and teeth.

Finally the f ntanelle was measured in mm on th radiograph.

The *Dawson preparations* were examined for bones which cannot be seen at all, or only with difficulty in the radiograph

1) metacarpus, 5 bones of which ne is very small (bones of the carpus are *not seen*), 2) phalanges, 14 in each forefoot, 3) tibia + fibula, are seen as one bone, which is, however divided into two bones t the proximal extremity 4) two bones in the tarsus calcaneous and astragalus, the remainder are not seen, 5) metatarsus, 4 bones, 6) phalanges, 12 in each hindfoot, 7) ribs, counted from the pleural sid special care has been taken to determine whether they are parallel, i e. whether there is any fusion, 8) bones of the skull, including the size of the fontanelle, length and width in mm, 9) fourteen t sixteen caudal segments. When inspecting the radiograph t is noted whether it is the third fourth sacral segment which is situated opposite the space between the two caudal nd the two cranial pelvic bones. W ca then determine which segment in the correspondi g Dawso preparation, should be designated the first caudal segment. The caudal segments can be counted exactly as even quite mall skeletal parts, as already mentioned, are distinctly stained

Table 3 shows some of the results of the countings nd measurements.

Histological examinations

As it is well known that phenylbutazone may cause lesions of the wall of the stomach when administered subcutaneously to nimals (Tarnoc et al 1957 Domenjoz 1960), we vainly ttempted t find such lesions the foetuses f the rabbit without, however examini g them histologically On the other hand, we made histological examination f the effect of the three drugs on the stomach f the females. It must, however be remembered that the does were treated from the first to the twentieth day inclusive after mating, whereas the *Caesarean section was first performed on the thirtieth t thirtyfirst day* Possible improvement or recovery may have taken place the period of 11 days between the withdrawal of the drug and the performance of the Caesarea section.

The following histological diagnoses ere used

Incipient erosion, meaning an exfoliation of the surface columnar epithelium accom*panied by haemorrh* ges and scattered polymorphonuclear leukocytes the deeper parts of the mucous membrane keeping in mind that this is lesion which occurs intravitally

Erosion, meaning exfoliation of parts f the mucous membrane but not to its f ll depth On the surface, fibrin nd possibly clots are seen, and addition, necrosis nd infiltration with polymorphonuclear leukocytes may be present in the remaining part of the membrane.

Chronic ulcer a change which the mucous membrane becomes exfoliated its full depth nd in hich the muscularis mucosae may also be bsent. The base of the defect is covered by layer of necrotic tissue infiltrated th polymorphonuclear leukocytes under which there is zone of granulation tissue ith numerous blood vessels, polymorphonuclear leukocytes, plasma cells and lymphocytes, and in addition proliferation of the connective tissue in the adjacen tissues to arying degree, frequently extending int the muscularis

This formation of scar tissue may be very marked at the edges of the ulcer which are raised and tumour-like.

The histological examination of the material was made without the investigator (E. B.) having any knowledge of which treatment had been given to the different groups. The result of the histological examination of the stomachs of the does is shown in Table IV

Results

A survey of the experiment and of the fertility etc. is given in Table I In Table 2 with the accompanying "key to Table 2" the details of the experiment are given, though the number of ribs and caudal vertebrae as well as the size of the fontanelles are given in Table 3 The result of the histological examinations of the stomachs of the does is given in Table 4

Discussion

It can be seen from Table I that only thalidomide definitely reduced *fertility* and the average weight of the young. The 6 animals in group D that received the large dose of 150 mg monophenylbutazone per kg body weight daily gave birth to 45 young as compared to 48 young by 8 females (6) in group F

Twenty less young were bred in the phenylbutazone groups *A + C than in the* groups B + D (monophenylbutazone). However it would only have been possible with certainty to establish that Phenylbutazone had reduced the fertility if a far greater number of dead foetuses and foetal remnants had been found in the females belonging to group A + C than in group B + D From Table 3 it can be seen that such a higher death rate among the embryos was not found in group A + C. (On the other hand, a higher death rate of the foetuses is seen in group E)

Since all 24 does in groups A, B C and D bred young, the administration of thalidomide must be held responsible for 4 out of 8 females not breeding in group E particularly as the finding of spermatozoa in the vaginal smears from these 4 females, taken after mating, showed that mating had occurred. There were, however also in group F i. e. the negative control group females (2) which did not breed and which cannot be accounted for as the sterile solution of glucose was the same as that administered to the above mentioned 4 groups. These infertile females had previously bred 8 litters each, and the males with which they were mated had been shown to be fertile by mating with other females in this investigation. The infertility can thus not be explained.

The average weight of the young in groups B and D is not low when taking into consideration the large number of young and the fact that they were taken from the dam on the 30th day after mating, while the young

Table 1

Embryotoxic experiments with doe rabbits. Fertility and weight of live young delivered by Caesarean section on the 30th to 31st day after mating.

Group	Daily dose per kg rabbet (from the 1st to the 20th day after mating (including Sundays) by subcutaneous injection in the back	Number of does in the group	Size of litters, (number of young)	Number of live young in the group as a whole	Average weight and variations in weight (S.D.) in the different *litters* (average with due consideration to the size of the litters).
A	30 mg phenylbutazone in 10 ml.	6 does	9+5+6+2+2+8	32 young	51 g ± 16.3 % (S.D.)
B	60 mg monophenylbutazone in 10 ml	6 does	7+9+3+6+7+10	42 young	49 g ± 12.1 % (S.D.)
C	60 mg phenylbutazone in 10 ml	6 does	6+6+7+7+3+6	35 young	53 g ± 15.5 %
D	190 mg monophenylbutazone in 10 ml	6 does	4+9+8+7+7+10	45 young	48 g ± 16.6 %
E	60 mg thalidomide in 1 ml	8 does	0+2+0+2+6+3+0+0	13 young	42 g ± 9.8 %
F	10 ml pure solvent	8 does	8+10+8+8+8+6+0+0	48 young	50 g ± 9.3 %

Table 2

The result of the gross examination of the exterior and interior of the young given in details.

Group designation. Doe rabbit treated with	No. and weight (in kg) of doe rabbit	Normal birth (norm.) or Caesarean section (c.s.) days after mating	Dead foetuses (f.) or remains of foetuses or remainders of placentae in the uterus	Sum of live young, normal or abnormal	Details bout the young
A	539 3.80	c.s. 31	0	9	normal1)
30 mg phenylbutazone d lly from the 1st to the 20th day after mating	615 3.60	c.s. 31	4 grey remnants of placenta	5	1 of the young normal. 2 of the young' right eye slightly open, otherwise normal. had umbilical hernias, ne of them to a marked extent
	393 4 10	norm. 31	?	6	1 of the young disappeared 5 young with a normal appearence were allowed to live. They thrived well.
	699 3.70	norm. 32	?	2	1 of the young normal, 36 g, died of unknown reason The other one also normal, was llowed to live and thrived well
	604 4 60	c.s. 31	2 grey remnants of placenta	2	normal
	592 3 45	c.s. 31		8	normal
B	758 4.20	c.s. 30		9	normal
60mg monophenylbutazone daily from the 1st to the 20th day after mating	478 3 60	c.s. 30		7	normal
	339 4 60	c.s. 30		3	normal
	790 4 20	c.s. 30		6	normal
	711 4 50	c.s. 30		7	6 normal. The 7th see item 20) in key to table 2
	16 4.50	norm. 31		10	5 days after birth 5 of the young were killed, average weight 52 g, normal the other 5 were llowed to live. They thrived well and were still al ve 45 days after birth.
C	722 3.80	c.s. 31		6	normal
60 mg phenylbutazone daily from the 1st to the 20th day after mating	393 4 10	c.s. 31		6	normal
	656 4.30	c.s. 31		7	1 of the young had an umbilical hernia with intestinal loops and a lobe f the liver in it the other 6 were normal.
	595 4.40	c.s. 31		7	6 normal. The 7th See item 10) and 21)
	483 4 10	c.s. 31		6	5 normal. The 6th item 11) and 22)
	742 3 70	norm. 31		4	1 f the young that had not been examined disappeared shortly after birth. 3 other young were normal and were allowed to live. They were still alive 45 days after birth.

1) Normal" means normal exterior and interio t the gross examination. Normal" used about young rabbits that were allowed t live means normal exterior at the gross examination.

Group designation. Doe rabbit treated with	No. and weight (in kg) of doe rabbit		Normal birth (norm.) or Caesarean section (c.s.) days after mating	Dead foetuses (f) or remains of foetuses or remainders of placentae in the uterus	Sum of the young, normal or abnormal	Details about the young
D	133	4.20	norm. 31		5	1 of the young that had not been examined disappeared. 4 other young were normal and were allowed to live. Were still alive 45 days after birth
150 mg monophenylbutazone daily from the 1st to the 20th day after mating	508	4.05	c.s. 30	2 dead f (20+2. g)	9	9 live young, normal
	703	4.50	c.s. 30	1 f nearly absorbed	8	8 normal
	115	4.20	c.s. 30		7	normal
	333	3.90	n. 30		7	normal
	409	4.40	n. 30	1 dead f. (23 g)	10	In 4 young, blood (clots) was found around bladder otherwise normal. 6 normal
E	66	4.45	31	0	0	
60 mg thalidomide daily from the 1st to the 20th day after mating	16	4.60	c.s. 31	8 f. nearly absorbed	2	1 young with the following abnormalities 4) and 11), otherwise normal. The other young one 5), 9), 11) and 23)
	518	4.30	c.s. 31	0	0	
	552	4.00	c.s. 31	4 dead f partly absorbed	2	in one the lower part of the spine seemed to be missing, in addition 6), 7), 10) 14), 15), 17), 18), 19) and 24) The other young one 2), 12), 15) and 16).
	280	3.90	c.s. 31	0	0	
	628	4.30	n. 31		6	1st of the young 3) 10) 19) 25). 2nd 11) 13) 19) 26) 3rd 11) 19) 27). 4th 11) 12) 19) 28). 5th 19) 6th 19)
	615	3.90	31		3	1st of the young 4) 11) 19) 29), 2nd 5). 3rd 5) 8) 11) 13), the latter pronounced, 30)
	224	4.10	n. 35 [1]	0	0	
F	343	4.30	c.s. 31	1 dead f	8	7 of the young quite normal. One had perhaps a dilatered colon, otherwise normal
10 ml 3% solution of glucose daily from the 1st to the 20th day after mating	253	4.20	n. 31		10	normal
	545	4.20	31		8	normal
	584	3.80	n. 31		8	normal
	345	4.70	c.s. 1	1 dead f.	8	normal
	293	4.20	norm. 1		6	normal, were allowed to live; still alive 45 days after birth
	667	3.80	c.s. 32	0	0	
	318	3.90	c.s. 32	0	0	

[1] It was intended that no. 224 should have bred normally

in the other 4 groups were taken on the 31st dav see Table 2. The young in the control group F are evidently the most uniform in size (S D = ±9 3 %)

Table 2 gives the details of the contents of the uterus of the participating does and of the results of the gross examination of the young as regards both exterior and interior examination In the key to Table 2 it can be seen that there were no cases of enlarged spleens, a phenomenon observed by THORPE (1964) after treatment of a patient with phenylbutazone

Umbilical hernia is also observed in the young of rat does treated with reserpine or similar substances (GOLDMAN & YAKOVAC 1965)

Table 3 shows that most of the young rabbits in the control group had 24 ribs, but this not was so in groups C and D The 25th and 26th rib are nearly always quite small so their occurrence or absence must be considered as insignificant

Only in group E is the average number of caudal vertebrae markedly reduced and the fontanelles larger than normal The size of the fontanelle can not evidently be estimated from the radiographs, but these do possibly give some information about the thickness of the skull at the edge of the fontanelle Only the Dawson preparations give the exact size of the fontanelle.

It appears from Table 4 that the most marked changes are seen in group A and group C comprising the animals treated with phenylbutazone in two different doses The changes in group B and group D treated with monophenvlbutazone also in two different doses, are less marked, but more pronounced in group D than in group B In group E, the thalidomide group, only slight changes are found. On the other hand, neither erosions nor chronic ulcers are found in group F the control group

Table 5 gives a synopsis of the results given in Table 2. It appears that with the doses used, there is no great difference in *foetal mortality* between the phenylbutazone groups, the monophenylbutazone groups and the control group, while this is significantly greater in the thalidomide group.

With Regard to *anomalies* it can be seen, as was to be expected, that they are much more frequent in group E than in any of the other groups There is some difference between groups (A + C) and groups (B + D) in both cases anomalies are found in the skeletal system whereas anomalies in the soft tissues are only definitely seen in the phenylbutazone groups There were, however in group D 4 young rabbits with clotted blood around the urinary bladder The cause of this has not been found in the present experiment. However in a later experiment 2 young rabbits, delivered by Caesarean section on the 30th day from two different normal, i. e. untreated females, were found to have strongly dilated umbilical arteries about 3 mm in width in the whole of their visible length

Table 5

Survey of dead foetuses, foetal remnants etc. and of *anomalies* found in the young rabbits Taken from Table 2 with accompanying Key

Group	Dead foetuses, foetal remnants etc.	Anomalies in the living young
A 30 mg phenylbutazone	I 2 rabbits altogether 6 remnants of placenta	4 of the young abnormal: 2 with an open eye, 2 with umbilical hernias
B 60 mg monophenyl butazone	No sign f dead foetuses	1 of the young abnormal with fusion of ribs on both sides and bnormal vertebral column i the thoracic part
C 60 mg phenylbutazone	N sign of dead foetuses	3 young bnormal 1 with umbilical hernia, 1 with bnormal sternebrae, only 3 sacral segments and no caudal segments, and 1 w th only 3 caudal segments
D 150 mg monophenyl-butazone	In 3 rabbits altogether 4 dead foetuses	4 young with clotted blood round the urinary bladder possibly an rtefact, see text
E 60 mg thalidomide	I rabb ts 12 dead foetuses foetal remnants	11 young (13 ?) with numerous bnormalities, see T ble 2 and accompanying key as w ll as in T ble 3
F Control group	In 2 rabbits altogether 2 dead foetuses	Rectal end of the colon distended in one young rabbit

from the neck of the bladder to the umbilicus, probably as a result of the operation If such a dilation had progressed further still it is possible that a haemorrhage from these vessels might have occurred with a resulting perivesicular haematoma such as was found in the above mentioned 4 young rabbits.

In group F only one rabbit with a possibly unsignificant colon dilatation was found Because of the otherwise normal conditions in the latter group, it can be concluded that phenylbutazone and monophenylbutazone in all probability have some embryotoxic effect. The lesions are, however not extensive in view of the large number of young (154 young rabbits in all in the 4 groups). In connection with the appearence of these lesions, the very large doses used per kg rabbit must be emphasized

The value of an experiment like the present one would be greater if the following conditions had been fulfilled that the two drugs in man and rabbit were metabolised by the same route, and that the rates of elimina

tion for the unchanged drugs and their metabolites were comparable in man and rabbit. The embryotoxic component or components would then be found in the blood of the two species in comparable concentrations for comparable periods of time. To clear up these questions would in itself entail much work.

That lesions of the stomach occur both in does and in human subjects indicates, that the same or a similar compound or compounds with injurious effect on the stomach, might actually be found circulating in the blood of the rabbit – for a sufficient period of time – as in man.

Summary

Experiments on embryotoxic effect in rabbits have been performed on 40 mated does divided up into 6 groups For the first 20 days after mating each doe received daily by subcutaneous injection

1) 30 mg/kg or 2) 60 mg/kg phenylbutazone,
3) 60 mg/kg or 4) 150 mg/kg monophenylbutazone,
5) 60 mg/kg thalidomide or
6) 3 / glucose solution.

The young were removed from the dam by Caesarean section on the 31st day after mating. The two phenylbutazones did not appear to reduce fertility Even if large doses of both substances were used, it is not possible to dismiss the possibility of an embryotoxic effect on the skeletal system, and on the soft tissues as far as phenylbutazone is concerned. The embryotoxic effects are, however not comparable with those of thalidomide. The two butazones, particularly phenylbutazone, had an injurious effect on the stomachs of the does, which were examined hi stologically

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